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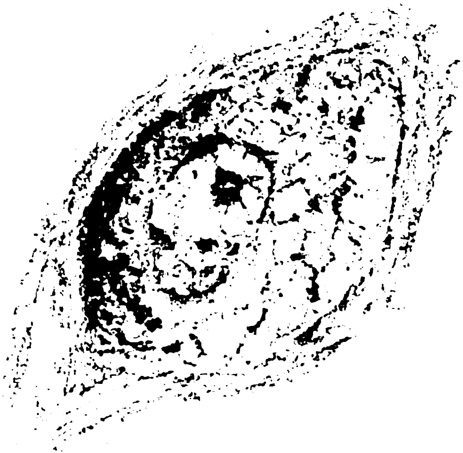

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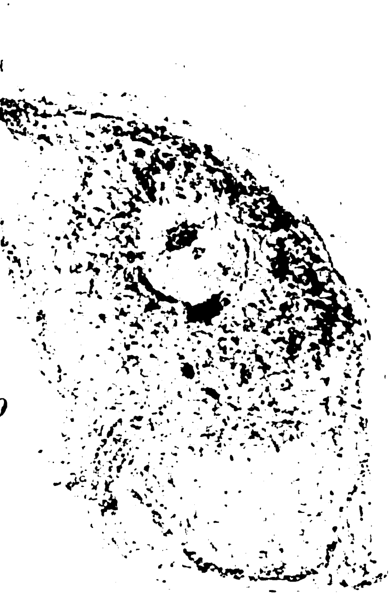

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THE JOURNAL OF MEDICAL RESEARCH

EDITED BY
HAROLD C. ERNST, M.D.

VOLUME XX.
(New Series, Vol. XV.)

JANUARY TO JUNE, 1909
Numbers 111, 112, 113, 114

BOSTON
MASSACHUSETTS
U.S.A.

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VOLUME XX., No. 1
(New Series, Vol. XV.)

JANUARY, 1909

Whole No. 111

THE
JOURNAL
OF
MEDICAL RESEARCH

Continuation of the
JOURNAL OF THE BOSTON SOCIETY OF MEDICAL SCIENCES

EDITED BY
HAROLD C. ERNST, M.D.

EDITORIAL OFFICE: 240 LONGWOOD AVENUE
BOSTON, MASS., U.S.A.

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THE
Journal of Medical Research.
(New Series, Volume XV.)

Vol. XX., No. 1. JANUARY, 1909. Whole No. 111.

IMMUNITY PRODUCTION BY INOCULATION OF INCREASING NUMBERS OF BACTERIA BEGINNING WITH ONE LIVING ORGANISM.*

Gerald Bertram Webb, M.D., and William Whitridge Williams, M.D.
(Of Colorado Springs, Col.)
In conjunction with Professor M. A. Barber.
(Of the University of Kansas.)
(From the Webb Tuberculosis Research Laboratory, Colorado Springs, Col.)

At the June, 1908, meeting of the Association for the Study and Prevention of Tuberculosis a brief report was read† relating some experiments in immunizing some mice against anthrax, and it was promised that similar experiments relating to the production of immunity against tuberculosis in guinea-pigs would later be reported.

Historical. — The use of the guinea-pig as a laboratory animal for the attempts at the production of immunity against tuberculosis has been common for many years. Mammalian and avian tubercle bacilli, living (attenuated or virulent) and dead, have been employed for immunity production, as well as bacterial products and so-called sera. Not one of countless experiments has established any certain efficiency. Such inadequacy led some to experiment with other acid-fast bacilli, resembling the tubercle bacillus, the butter, grass, and manure bacilli, as well as with the bacilli of cold-blooded animals and mammalian tubercle bacilli modified by their passage through such animals. Some results have shown that some immunity has been produced because of the length of life over the control animals of those inoculated after virulent tests, but in general no complete protection has been afforded.[1] Every species of animal is susceptible to certain bacteria and non-susceptible to others.

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* Read in Section I. International Congress on Tuberculosis, Washington, Oct. 1, 1908. Received for publication Oct. 21, 1908.

† "Immunity production, by inoculation of increasing numbers of bacteria beginning with one living organism." Preliminary report, by Gerald Bertram Webb, M.D., and William Whitridge Williams, M.D., of Colorado Springs, Col.

A knowledge of the principle that one attack of an infectious disease confers some immunity belonged to the ancients, and the virus of diseases such as smallpox in man and rinderpest in animals was used for the production of immunity. Jenner was the first, however, to employ attenuated or weakened virus successfully, — the smallpox virus permanently modified by passage of the disease through the cow. Pasteur's work followed this idea, to prevent disease by protective inoculation of weakened virus, and beginning with cultures of chicken cholera, attenuated by accident, he invented new methods for reducing virulence, and attenuated anthrax bacilli by growth at temperatures higher than those to which they were accustomed, rouget in swine by successive passages through the rabbit, and hydrophobia by a long series of inoculations into rabbits. Some degree of artificial immunity has been produced for pathogenic bacteria by previous inoculations with some other organism, and Pasteur noted that attenuated chicken cholera bacteria would protect chickens against anthrax.

Artificial immunity has been attempted by inoculation of a small number of virulent bacteria, and Chauveau, working with diluted cultures and Algerian sheep, immunized them to anthrax, although the same dosage killed French sheep.

At first the interpretation of the influence of the numbers of bacteria injected met with incredulity. To-day, however, it is dogmatic. Arloing, Cornevin, and Thomas produced immunity in this way against symptomatic anthrax. Peugh inoculated without disaster the diluted virus of Claveléc (sheep pox). Charrin used the dilution method against B. pyocyaneus. In the manufacture of antistreptococcus serum and diphtheria antitoxin examples of this may be seen. Hutyra[2] was successful in repeatedly injecting a calf with virulent bovine tubercle bacilli, by beginning with two milligrams and increasing to one hundred milligrams in seventeen months.

The earnest efforts to immunize animals against tuberculosis began with the discovery of tuberculin by Koch[3] in 1890. Koch[4] later concluded that an antibacterial immunity as well as an antitoxic immunity was necessary for protection against, and the treatment of, tuberculosis, and finally produced T R, which was found to induce the highest immunity of any tuberculin. He found guinea-pigs could be protected against infection with virulent tubercle bacilli by previous treatment with T R. Others corroborated this, and also found it to contribute to prolonging the life of infected guinea-pigs.

The outcome of all this work has been to establish the fact that tuberculin — dead tubercle bacilli and their products — can have a specific effect on tuberculous lesions, and sometimes cause them to become encapsulated or even to disappear, and that although this effect is not constant, their proper usage seems to insure an average longer tenure of life, both with cattle[5] and with human beings.[6]

More and more, however, it became evident that with a disease which appeared to yield so low a grade of immunity as does tuberculosis, protection must be obtained against the living virus as well as against its poisons.

Probably the first work done with the living bacillus was that of Dixon,[7] with attenuated cultures. He found that animals (guinea-pigs, rabbits, opossums) inoculated with an old culture containing club-shaped and branching forms of tubercle bacilli would resist subsequent inoculation with virulent organism. Trudeau[8] in 1892–3 found that by subcutaneous preventative inoculation of living cultures of avian tubercle bacilli he was able to increase the resistance of a rabbit to infection of living virulent mammalian cultures. His[9] remarks ten years later, quoted by Pearson,[10] are, as we view this subject, of especial interest. "It was only when I began to make use of living cultures as a protective inoculation that I met any encouraging results, and my experience would indicate that the living germ is essential to what success has been attained in the production of artificial immunity against tuberculosis." De Schweinitz,[11] in 1894, with attenuated cultures of human tubercle bacilli, a twentieth generation on glycerine beef broth of slightly acid reaction, immunized guinea-pigs so that they remained well following the injection of tuberculous material from a cow, when control animals died with tuberculosis in seven weeks. De Schweinitz also injected, in increasing doses, human tubercle bacilli into cattle, and obtained toleration. McFadyean[12] concluded from bovine experiments that "whatever may have been the degree of natural immunity possessed by these three experimented cattle, it was much increased by the successive intravenous inoculations to which they were subjected. The immunity was not absolute, but it may be doubted whether a degree of resistance that will merit this term is obtainable by any method in cattle." E. Levy[13] immunized guinea-pigs against tuberculosis by giving several inoculations of tubercle bacilli, weakened by glycerine. Pearson and Gilliland[14] showed that the intravenous injections of living human tubercle bacilli were harmless to cattle, and would protect these animals against subsequent inoculations with the bovine bacilli, and were the first to prove that the systematic preliminary treatment with human tubercle bacilli alone would procure this immunity. Von Behring[15] and also Thomassen[16] have obtained similar results, and to Von Behring belongs the credit of first proposing a definite method for the practical application of vaccinating cattle against tuberculosis. Neufeld,[17] after thorough trials, abandoned attempts to immunize experimental animals with toxins and dead tubercle bacilli, and produced immunity by the use of living attenuated tubercle bacilli of both human and bovine type. He also pointed out the possibility of over-vaccinating, especially in goats. Too large doses of human tubercle bacilli produced little immunity. Neufeld usually preceded the injection of living organisms with injections of pulverized bacilli. The most extensive experiments to ascertain if the human tubercle bacillus could bring about curative effect over existing early lesions of tuberculosis were made by Pearson and Gilliland.[18] Theobald Smith,[19] in quoting their work on calves, writes that the evidence presented by their experiments goes to show that it is possible to arrest tuberculosis, but not to rid the animal of living bacilli. Theobald Smith has succeeded with a carefully tested attenuated bovine culture in obtaining, in a single injection, as good results as were obtained by double vaccination with living human bacilli. Similar work has been done by Koch[20] and his co-workers.

Importance of using the living virus. — Sustained by the results in diphtheria, the hope that all infectious diseases would be combatted by the use of antitoxins has not been realized, and investigators are returning to the use of vaccines for the prevention and cure of these diseases.

By means of cultures of typhoid bacilli, killed in a manner which least harms the protoplasm, Wright[21] has obtained a marked degree of protection to typhoid. This success unfortunately has not accompanied similar attempts with other diseases, although a good measure has been reached therapeutically in the treatment of some infections (staphylococcus, gonococcus).

In addition to other failures in the production of immunity to tuberculosis with dead bacilli must be mentioned Theobald Smith's[19] experience. Bovine cultures killed at 60° C. for from forty to sixty minutes, and then inoculated in the strength of five to ten times that of living human tubercle bacilli which had given immunity in cattle to the bovine bacillus, failed to produce any appreciable resistance in the experimental animals.

The[22] discovery of healed or healing tuberculous foci in seventy to ninety per cent of all autopsies, in contrast to the fifteen to twenty per cent of deaths from tuberculosis shows that susceptibility and immunity are subject to marked individual variations.

The ability of an individual to overcome a tuberculous infection is referred in a vague way to an unusual resistance on his part, and the bacilli may have gained foothold during a temporary decline of this resistance. The great majority of mankind must have, in some degree, a natural immunity against tuberculosis. Were it not so, the race would long ago have become nearly extinct. From what we are learning from the opsonic index, this natural immunity is sometimes fortified by subsequent autoinoculation in early infection, resulting in the healed lesions.

It is established that vaccination of calves with the human type of the tubercle bacillus is harmless, and gives them a relatively high resistance to fatal doses of the bovine variety. The immunity produced, however, has not appeared entirely satisfactory in regard to degree and duration, and should probably be fortified by a subsequent injection of bovine bacilli.

To the success in smallpox and in rabies in which attenuated virus has proved successful must lately be added the work of Strong[23] in immunization against plague.

The whole story of immunization has led investigators to expect a solution of tuberculosis immunity through the use of living virus.

Should the necessity for the introduction of the living virus in tuberculosis be granted, the natural question arises, "What form will it take, and by what route shall it be introduced?" Let us consider the last question first.

It is known that the same dose of a virulent culture or vaccine can cause death or simply protect according to the site selected for introduction of the organism.

Roche-Lubin observed that the process of immunizing to sheeprot by feeding was less deadly than by the lancet method. Forty years ago cattle were immunized against "peripneumonia" by the procedure of Willems, which consisted in introducing serum from the pulmonary lesions into the end of the tail. It was found that the local tumefaction was slight and that death was not nearly so frequent as when the inoculation was made into the main body. Chauveau immunized cattle to the same disease by injecting directly into the veins. Chauveau and Arloing found that intravenous injections of Bacillus septicus immunized donkeys and dogs, whereas subcutaneous inoculations were fatal. Attempts to immunize cattle by feeding tubercle bacilli have been made by Calmette[24] and Guerin, who also produced some degree of immunity in guinea-pigs by feeding to them certain cold-blooded tubercle bacilli.

Most bovine immunization has been by intravenous injection. Several experimenters, however, have tried subcutaneous injection. Baumgarten,[25] Lignières,[26] and Hutyra[27] favor this method. Theobald Smith[28] very correctly makes the suggestion that it is of importance to ascertain if the lungs, the most frequently affected organs, in the spontaneous disease, and which receive most of the vaccine by the intravenous method are as well protected by the one as the other method.

In all our work the subcutaneous has been the route employed, and in no case has there been formed at the place of injection any permanent lesion, either tumor or any focus discharging tubercle bacilli.

And now to consider the question as to what form the virus must take so that safe immunization can be achieved.

All conceivable forms of attenuation of tubercle bacilli cultures have been tried, and of these attenuated cultures minute amounts by weight have been inoculated, and we think perhaps the result of part of our work and that of others we will quote will explain why all these methods have failed.

The work of Gebhardt[29] suggests that the inhalation of four hundred tubercle bacilli can cause tuberculosis in a guinea-pig. Preyss[30] found on one occasion the inhalation of only thirty-six bacilli were necessary, and another time forty-eight. Lately Findel,[31] with more accurate methods of enumerating the numbers inhaled, places the number at sixty-two. But he found that even twenty bacilli could produce infection, and makes the suggestion that possibly only one bacillus is sufficient to infect young guinea-pigs.

Findel also concluded that the mortal dose taken by the digestive tract would be at least six thousand times the number inhaled. Ziesché[22] in reviewing this work is of the opinion that it is more than probable that everything being taken into consideration we must allow the same number, although it perhaps may be more, inhaled to be able to infect man. Flügge[22] agrees with this.

The virulence of the bacilli would need some consideration in making absolute deductions, but the point we wish to emphasize from these investigations is the few bacilli which are necessary to bring about infection.

One of our results will show that five tubercle bacilli, followed a few days later by fifteen — in all, twenty bacilli — produced tuberculosis, following subcutaneous inoculation in a guinea-pig. One hundred and fifty bacilli of a culture more attenuated produced a similar result.

Wyssokowicz[23] has stated that eight tubercle bacilli have been able to set up an infection in the peritoneal cavity of the guinea-pig and that twenty-four to thirty are required in the rabbit.

It would scarcely, then, be possible to bring about such attenuation for immunization that amounts by dilution or weight could be safely used unless such doses were accurately counted.

In a true vaccination the virus should multiply in the tissues after inoculation. Strong[23] found in the production of immunity against plague that the organisms after inoculation had reproduced themselves in the tissues for probably one hundred or more generations within twenty-four hours. This initial growth of bacteria after inoculation, and before the organism prevails over them, is perhaps the chief cause of superiority of the use of the living over dead bacteria.

Welch[*] long ago suggested that the toxines necessary to produce immunity in some diseases are not elaborated by the bacteria when growing on artificial media, but only within the living body in response to the stimulus of the protective substances secreted by the living cells, which attempt the destruction of the invaders.

---

[*] Dr. Welch's hypothesis: "Looked at from the point of view of the bacterium, as well as from that of the animal host, according to the hypothesis advanced, the struggle between the bacteria and the body cells in infections may be conceived as an immunizing contest in which each participant is stimulated by its opponent in the production of cytotoxins hostile to the other, and thereby endeavors to make itself immune against its antagonist."

We have not been able to study the question as to whether growth of the tubercle bacilli took place subsequent to inoculation.

The rate of growth of what will be designated "Culture B," a somewhat attenuated culture, however, was studied.

Into six microscopic drops of glycerine veal broth single bacilli were introduced, and after fifteen hours incubation five out of the six had doubled, and the sixth bacillus had multiplied into three bacilli. These bacteria were briefly exposed to the light from an Argand gas burner, which illuminated the microscopic field in all our work.

This experiment, apart from demonstrating the rapidity of reproduction, also gave us satisfactory evidence that our bacilli were alive.

The rate of growth in the tissues of the guinea-pigs, however, cannot be estimated from this result, but the chances are probably against reproduction when only one bacillus has been introduced.

Experimental technic. — Emulsions were first made from the cultures, and bacteria were selected from hanging drops of these, according to the method of Barber.[34]

Inoculations were made direct from the isolating pipette, either by introducing the capillary end through a needle puncture of the skin or else by drawing back the fluid containing the bacteria further into the tube, and when at a safe distance making an inoculating point, with a peep flame, which could easily be inserted.

Frequently additional broth would be introduced back of that containing the bacteria, to insure a thorough flooding out of the contents.

In all cases of anthrax work, plain broth, and in tubercle work, glycerine veal broth, were used.

There is very little doubt as to the inoculation of the single bacterium, as some of the experiments themselves will indicate. Moreover, single bacteria have been selected and placed and stained on an ordinary glass slide selected and ejected as in the inoculations. These have been stained and found and examined with the oil immersion lens.

From other cultures, such as typhoid, single bacteria have been isolated and new cultures started from them.

Single cocci have been worked with and a single influenza bacillus successfully isolated.

In the use of small numbers, the error in counting was exceedingly slight, and the error in counting the higher numbers, although greater, was still inconsiderable.

For quite large doses (many thousands) the emulsions might be standardized by Wright's methods for counting vaccine.

All mice were inoculated subcutaneously at the root of the tail; almost all guinea-pigs subcutaneously in the abdominal region; all rabbits subcutaneously in the back.

Table I.

Anthrax Experiments.

Mice.		
Animal Number.	Initial Inoculation.	
1	1 thread.	2 days, dead, no evidence of anthrax.
2	1 thread.	4 days, 2 threads; 8 days, 3 threads; 12 days, 12 threads.
3	1 thread.	3 days, 2 threads; 12th day, 12 threads.
4	2 threads.	8 days, 12 threads.
5	3 threads.	Escaped 4th day.
6	4 threads.	Gave birth 4th day to 7 young. Escaped 5th day.
7	3 threads.	12 threads, 5 days.
8	3 threads.	12 threads, 5 days.
9	6 threads.	12 threads, 4 days; 25 threads, 9 days. Dead, anthrax bacilli in tissues.
10	6 threads.	12 threads, 4 days.

TABLE I. — *Continued.*

Mice.

Animal Number.	Initial Inoculation.	
11	6 threads.	12 threads, 4 days.
12	6 threads.	12 threads, 3 days; 50 threads, 9 days.
13	12 threads.	
14	1 loopful 20-hour broth culture, injected by same technic.	2 days, dead, anthrax bacilli in blood and spleen. Cultures made.
15	100 threads, culture from 14.	
16	250 threads, culture from 14.	Dead, anthrax bacilli in blood and spleen. Cultures made.
17	150 threads, 3d generation from 14.	
18	200 threads, 3d generation.	
19	.04 cc. emulsion in broth, same technic.	Dead, anthrax bacilli recovered for culture.
20	1 (18μ) thread direct from 19 blood.	3 days, dead, anthrax bacilli in tissues.
21	1 (57μ) thread direct from 19 blood.	3 days, dead, anthrax bacilli in tissues.
22	1 (3μ) thread from 19 culture.	
23	1 thread with 12 spores from 19 culture.	14 days, 68 spores, with about 14 rods. 17 days, dead, anthrax bacilli in tissues.
24	20 (483μ) threads from 17-hour agar culture, from 1st generation from mouse dead of anthrax.	
25 ...	1 (18–21μ) thread from heart's blood of dead mouse, after washing in salt solution.	5 days, dead, anthrax bacilli in tissues.
26	1 (16μ) thread from heart's blood of dead mouse, after washing in salt solution.	5 days, dead, anthrax bacilli in tissues.
27	27 (666μ) threads from 17-hour agar culture from 1st generation from mouse dead of anthrax.	5 days, 1 (18–24μ) thread directly from heart blood dead mouse.
28	70 spores and fragments grown in blood of infected mouse, washed in salt solution.	4 days, dead, anthrax bacilli in tissues.
29	9 spores and few degenerate rods, washed in salt solution, 24 hours, from blood of infected mouse.	5 days dead, anthrax bacilli in tissues.

When not otherwise stated it is to be understood that the animal survived.

It will be noticed that one thread (3–6 bacilli), isolated direct from blood of a mouse dying from anthrax, on four distinct occasions caused death when inoculated into a fresh mouse.

Nos. 22, 24, and 27 also survived eating the contents of the thorax of No. 28, following death from anthrax.

One thread (18μ) means one thread eighteen micromillimeters long, approximately five or six organisms.

One of these threads was isolated and stained on the slide, and the number of segments counted.

Apparently the minimum lethal dose lies between 6μ and 18μ, and lies below nine spores grown in the blood of an infected mouse.

Mouse No. 27 evidently obtained some immunity from the first inoculation, resisting one thread ($18–24\mu$) obtained directly from the heart blood of mouse dead with anthrax. One young rabbit was inoculated with three threads direct from peritoneal fluid of dead mouse 19 and survived.

We are allowed to mention some more recent work done by Professor Barber since leaving our laboratory:

No.	First Inoculation.	Further Inoculation with Intervals.
1	Lethal dose, death after 2 days.	
2	1 thread (6μ), death after 2 days, anthrax bacilli in organs.	
3	1 thread (9μ), interval 6 days.	1 (21μ), interval 10 days, 1 (40μ), alive 13 days after last inoculation.
4	1 thread (30μ), interval 7 days.	1 (18μ), interval 9 days; 1 (40μ), alive 13 days after last.
5	1 spore, interval 7 days.	2 spores, interval 8 days; 3 spores, interval 8 days; 5 dried spores, alive 4 days after last.
6	1 spore, interval 7 days.	4 spores, interval 7 days; 8 spores, interval 8 days; 12 dried spores, alive 4 days after last.
		(Nos. 5 and 6, last inoculation dried spores used.)
7	2 spores, after interval 4 days.	Anthrax bacilli found in tissues after death.
8	4 spores, after interval 4 days.	" " " " " " "
9	20 spores, after interval 3 days.	" " " " " " "
10	35 spores, after interval 2 days.	" " " " " " "
11	3 spores, interval 12 days.	6 spores, alive after 12 days.
12	14 spores, interval 12 days.	20 spores, alive after 12 days.
13	1 (9μ), interval 10 days.	11 (27μ), dead after 4 days.
14	1 ($7\frac{1}{2}\mu$), interval 10 days.	11 (27μ), alive after 13 days.

No.	First Inoculation.	Further Inoculation with Intervals.
15	4 spores, dead after 9 days, anthrax.	
16	I (6μ), alive after 22 days.	
17	I (21μ), interval 13 days.	III (84μ) plus 2 spores, alive after 7 days. (Here growth in hanging drop used first.)
18	I (36μ), interval 13 days.	IV. (84μ) plus 2 spores, alive after 7 days. (Here growth in hanging drop used first.)
19	I (12μ), alive after 21 days.	
20	I spore, alive after 20 days.	
21	I spore, died after 11 days. No anthrax found in blood or tissues.	
22 .	I spore, alive after 20 days.	
23	2 spores, alive after 20 days.	
24	I spore, alive after 18 days.	
25	3 spores, alive after 18 days.	
26	4 spores, alive after 18 days.	
27	I (12μ), alive after 17 days.	
28	I (6μ), alive after 17 days.	
29	Lethal dose apparently from blood of guinea-pig, alive after 17 days.	
30	2 spores, alive after 16 days.	
31	Lost.	
32	I (36μ).	
33	Lethal dose, died, 1 day.	
34	I (12μ), dead after 3 days.	
35	I (9μ), alive after 13 days.	
36	I (12μ), alive after 13 days.	
37	II (57μ), alive after 13 days.	
38	Lost.	
39	I (9μ), alive after 10 days.	
40	II (57μ), alive after 10 days.	
41	Large dose, degenerated rods, alive after 8 days.	
42	2 spores in mother cells, alive after 17 days.	
43	3 spores linked together in mother cells, alive after 7 days.	
44	4 spores linked together in mother cells, alive after 7 days.	
45	III (93μ) plus 2 spores of first growth in hanging drop, alive after 7 days.	
46	II (57μ) plus 3 spores of first growth in hanging drop, alive after 7 days.	
47	15 spores, dried, kept in refrigerator about 10 days before inoculation. Same lot of spores of which 4, when fresh, were fatal to No. 13. These spores proved capable of germination. No. 47 is alive after 4 days.	
48	4 spores like those of 47, alive after 4 days.	
49	I spore like those of 47, alive after 4 days.	
50	75 to 100 spores like those of No. 47, alive after 4 days.	

Unless otherwise stated, the rods used were taken directly from heart's blood of infected animal, and the spores used were grown in hanging drops in blood of infected animal, plus salt solution or broth. Spores formed usually less than twenty-four hours after starting culture, and temperature of 37° C. used. In all deaths except 21, anthrax bacilli were found in the blood.

ANTHRAX.

Guinea-Pig Series.

Guinea-pig A. 5 spores (same lot of which killed mouse No. 15), dead after 5 days, anthrax.

Guinea-pig B. 1 (29μ), interval 5 days; 11 (51μ), alive after 17 days.

Guinea-pig C. 1 (15μ), directly from blood of guinea-pig A, alive after 17 days. Showing a greater degree of immunity than the mouse.

From the foregoing work, it is evident that we have nothing to show that it is possible to immunize mice with virulent anthrax by graduated doses if bacteria are taken direct from the blood of a mouse dying of anthrax, but the attenuation produced by twelve hours on agar of these same bacilli allows it.

TABLE II.

Rabbits inoculated with human tubercle bacilli.

Rabbit Number.	1st Day.	3d Day.	5th Day.	9th Day.	15th Day.	Section.
1..............	1 T.B. (A).	5 T.B. (A).	....	25 T.B. (A).	100 T.B. (A).	90th day, no evidence of tuberculosis.
2..............	1 T.B. (A).	....	....	5 T.B. (A).	....	90th day, no T.B.
3..............	25 T.B. (A).	....	45 T.B. (A).	....	....	86th day, no T.B.
4..............	25 T.B. (A).	50 T.B. (A).	....	....	....	84th day, no T.B.
5..............	1 T.B. (B).	....	....	....	....	80th day, no T.B.
6..............	1 T.B. (B).	....	....	....	....	77th day, no T.B.
7..............	25 T.B. (B).	....	....	....	....	75th day, no T.B.
8..............	1,000 T.B. (B).	....	....	....	....	60th day, no T.B.

The work on rabbits was undertaken whilst awaiting a new consignment of guinea-pigs. Rabbits have been extensively used to test the immunizing and curative effects of various tuberculins, but we are aware they already possess a marked degree of immunity to the human tubercle bacillus.

Our experiments seem to corroborate this. Work, however, with the bovine bacillus, which will be of greater value, is being prepared for at the present time.

TABLE III.

Guinea-pig Series.

Number of Guinea-pig.	Initial Inoculation.	Section.
1	1 T.B. (1).	104th d, no T.B.
2	2 T.B. (1); 29th d, 10 (A); 34th d, 25 (A); 44th d, 75 (B); 55th d, 100; 63d d, 500; 75th d, 750; 85th d, 1,000; 89th d, 1,250; 95th d, 1,500. Total no. bacilli, 5,212.	110th d, no T.B.
3	5 T.B. (A); 4th d, 15 (A). Total no. bacilli, 20.	83d d, T.B. in inguinal glands.
4	5 T.B. (A); 4th d, 15 (A); 9th d, 50 (A); 18th d, 150 (B); 29th d, 250; 37th d, 500; 68th d, 700. Total no. bacilli, 1,670.	68th d, following abortion, no T.B.
5	25 T.B. (A); 4th d, 50 (A); 9th d, 100 (A); 18th d, 200; 28th d, 300; 36th d, 525; 45th d, 800; 51st d, 1,000. Total no. bacilli, 3,000.	60th d, no T.B.
6	25 T.B. (A).	55th d, no T.B.
7	50 T.B. (A); 9th d, 100; 19th d, 200; 27th d, 500. Total no. bacilli, 850.	51st d, no T.B.
8	50 T.B. (A).	76th d, no T.B.
9	1 T.B.	Lost.
10	1 T.B. (+); 4th d, 5.	52d d, no. T.B.
11	1 T.B. (+).	Lost.
12	1 T.B.; 4th d, 5; 9th d, 25; 11th d, 50; 19th d, 150; 25th d, 300; 31st d, 600. Total no. bacilli, 1,131.	39th d, no T.B.
13	1 T.B; 4th d, 5; 9th d, 25; 22d d, 75. Total no. bacilli, 106.	Lost.
14	1 T.B.; 4th d, 5; 10th d, 60; 15th d, 150; 19th d, 300; 23d d, 450; 31st d, 600; 39th d, 750; 44th d, 900; 49th d, 1,050. Total no. bacilli, 4,266.	74th d, no T.B.
15	1 T.B.; 2d d, 10.	69th d, no T.B.
16	1 T.B.	37th d, no T.B.

TABLE III. — *Continued.*

Number of Guinea-pig.	Initial Inoculation.	Section.
17 ...	1 T.B.; 2d d, 5; 9th d, 10; 14th d, 50; 22d d, 100; 31st d, 200; 37th d, 300; 42d d, 400.	Lost.
18	1 T.B.; 2d d, 10; 9th d, 25; 17th d, 40; 24th d, 60; 31st d, 85. Total no. bacilli, 221.	39th d, no T.B.
19	1 T.B.; 2d d, 10; 9th d, 25; 14th d, 50; 23d d, 75; 31st d, 100; 37th d, 125; 42d d, 150. Total no. bacilli, 536.	61st d, no T.B.
20	1 T.B.	61st d, no T.B.
21	100 T.B.; 15th d, hard nodule at inoculation site, which later disappeared.	33d d, no T.B.
22	100 T.B; 10th d, 200; 14th d, 300; 23d d, 400; 30th d, 500; 36th d, 600; 41st d, 700. Total no. bacilli, 2,800.	52d d, no T.B
23	.8 cc. emulsion, culture (B).	
24	150 T.B.	62d d, lungs full of tubercles.
25	150 T.B.; 7th d, 300; 11th d, 450; 20th d, 600; 28th d, 750; 34th d, 900; 39th d, 1,050.	Lost.
26	1 T.B.; 5th d, 5; 11th d, 10; 19th d, 15; 28th d, 20; 34th d, 25; 39th d, 30.	66th d, no T.B.
27	1 T.B. (+); 5th d, 5; 14th d, 15; 19th d, 25.	26th d, no T.B.
28	1 T.B. (+); 5th d, 5; 11th d, 80; 21st d, 150; 30th d, 250; 34th d, 350; 40th d, 450. Total no. bacilli, 1,286.	65th d, no T.B
29	1 T.B. (+); 5th d, 5.	60th d, no T.B.
30	1 T.B. (*).	62d d, no T.B.
31	320 T.B.; 5th d, 1,000; 18th d, 2,000. Total no. bacilli, 3,320.	26th d, no T.B.
32 ...	300.	60th d, no T.B.
33	500; 12th d, 1,000; 22d d, 3,000; 27th d, 5,000; 33d d, 7,000.	Lost.
34	500.	22d d, no T.B.
35	500; 12th d, 2,500. Total no. bacilli, 3,000.	32d, no. T.B.
36	500; 12th d, 3,500; 16th d, 6,500; 22d d, 9,500; 29th d, 11,500.	General tuberculosis.
37	1,000.	Tubercles in spleen.
38 ...	1,000; 9th d, 1,500; 17th d, 2,000; 20th d, 2,500; 26th d, 3,000. Total no. bacilli, 10,000.	Tubercles in lungs and liver.
39	2,000.	16th d, death due to a septicemia lymphangitis, spreading from inoculation site.
40	2,000; 8th d, 3,000; 20th d, 4,000; 25th d, 5,000. Total no. bacilli, 14,000.	50th d, tubercles in lungs and liver.
41	3,000.	51st d, tubercles in lungs and liver.

TABLE III. — *Concluded.*

Number of Guinea-pig.	Initial Inoculation.	Section.
42	3,000; 7th d, 4,500; 15th d, 6,000; 20th d, 7,500; 25th d, 9,000. Total no. bacilli, 30,000.	51st d, tubercles in lungs, liver and spleen.
43	4,000.	48th d, tubercles in lungs and spleen.
44	4,000; 15th d, 6,000; 21st d, 8,000. Total no. bacilli, 18,000.	46th d, no T.B.
45	1 T.B. obtained from inguinal glands of No. 23 on the 27th day after inoculation of .8 cc. emulsion of culture B.	46th d, no T.B.

Professor Barber has since inoculated a guinea-pig with one tubercle bacillus taken direct from an infected hog. After thirty-eight days no lesion at autopsy.

In the interpretation of the above table "d" means day, T. B. means tubercle bacillus or tuberculosis, "(1)" tubercle bacillus isolated two months earlier from an active case of pulmonary tuberculosis, originally on Hesse's formula (Heyden's Nährstoff), from this to glycerine agar with egg yolk.

"(A)" indicates virulent tubercle bacillus culture H 39, obtained through the kindness of Dr. E. R. Baldwin, Saranac. It was isolated directly from sputum of an acute pulmonary case on Hesse's agar, Oct. 3, 1906, thence to egg tubes Oct. 16, 1906, thence to beef serum with monthly transfers since.

When not specified a culture of tubercle bacillus (B) obtained from Parke Davis was used. These gentlemen are not able to tell us the exact origin of this culture, but know it originated either from one supplied them by Dr. Theobald Smith or by Dr. Trudeau.

We preferred using culture "B" because it emulsified easier, and we were the easier able to avoid clumps.

Eight-tenths of a cubic centimeter of an emulsion of culture "B" was injected into a guinea-pig by the same technic as that employed in the rest of the work, twenty-seven days later tubercle bacilli were taken from an inguinal gland. The exact day of death, however, was not recorded.

(+) signifies the bacilli were especially selected on account of certain refringent characteristics. The bacillus given in No. 10 had a dot in one end. These bacilli usually contrasted strongly with their fellows.

(*) This bacillus was shaped like a spermatozoon.

From A and B fresh cultures were grown every few weeks on glycerine agar containing egg yolk, this being the medium on which they thrived best.

In not one of the pigs was a lesion at the inoculated site found at autopsy, and only once or twice could a nodule be felt following an inoculation. Several of our pigs died or were killed because of an epidemic gastro-enteritis. Those referred to as lost were unfortunately stolen from their cages. No pig apparently gave evidence of any condition resulting from anaphylaxis due to the repeated injections of the small amounts of glycerine veal broth in which the bacilli were conveyed. The reason for using glycerine veal broth in the pipette was to ensure the bacilli remaining alive.

The table of the guinea-pig series speaks for itself. The fact that No. 3 proved tuberculous following only twenty bacilli inoculated, although a solitary instance, indicates the necessity for beginning with one bacillus. Culture "A" was more virulent than culture "B." The minimum dose of culture "B" producing tuberculosis appeared to be one hundred and fifty bacilli. In all cases in which the initial dose was one bacillus the pigs remained free from tuberculosis, even receiving final doses of one thousand or one thousand five hundred.

In making the autopsies, smears were made from the inguinal glands usually, and stained by the Ziehl-Neelsen and Gram stains.

Sections were made for microscopic examination of suspicious areas in different organs.

The work of Lannelongue[40] suggests that microscopic examination is not sufficient to show absence of tuberculosis, and that different tissues should be injected into fresh guinea-pigs. We were not able to do this on account of scarcity of pigs.

EVIDENCES OF IMMUNITY FROM THE FOREGOING EXPERIMENTS. — From the work with mice and guinea-pigs a remarkable degree of individual susceptibility and resistance to anthrax and tuberculosis will be noticed. This variation in the degree of natural immunity to bacteria no doubt must be counted with in all experimental work with laboratory animals.

The fact that large doses — numbers many times beyond those possible to produce disease in individual animals — were safely inoculated and the consideration of the total number of bacteria altogether inoculated must indicate some degree of successful immunity.

Strong[23] found in immunizing monkeys against plague that when they are able to resist and survive over twelve times the maximum fatal dose, agglutinins could not be demonstrated in their blood. Only small quantities of agglutinins could be detected in the examinations of several test immune sera prepared from horses, and which were known to possess considerable protective power. Strong says "in conformity with the experiments of Kolle and Hetsch, and more recently Dean, I have found that plague immune serum which is known to possess anti-infectious power in the animal reveals in vitro no bacteriolytic action. I have been able to show that the blood serum of both human beings and animals vaccinated and immunized against pest frequently revealed an increased opsonic index although not invariably."

Our blood tests in the tuberculosis experimented animals resulted in a similar manner. With some of the guinea-pigs we undertook to attempt to estimate the amount of opsonin and lysin. The blood was procured direct from the heart, and high opsonic indices were found in guinea-pigs later shown to be decidedly tuberculous. For instance, No. 37 gave indices of 2 and 1.9, whereas guinea-pig No. 2 gave an index of 1.

The amount of lysin was estimated by what was suggested by one of us[41] as a possible lytic index method. The guinea-pigs were found not to vary, whereas men inoculated in a similar manner showed variations amounting to several times the lysin contained in normal sera, when their opsonin was normal.

It is known that phagocytosis in the guinea-pig occurs very readily, and it is perhaps possible that a guinea-pig and man depend upon a different principle of defence to tuberculosis. It may be recalled that in anthrax dogs owe their immunity to phagocytosis and white rats to lysis of the bacilli.

The possibility that subsequent inoculations may have been instrumental in clearing away beginning infection must not be forgotten, — in addition to individual natural resistance, — in the case of such guinea-pigs as No. 44.

The majority of our pigs weighed from three hundred to six hundred grams at the beginning of inoculations. Many although becoming tuberculous gained considerable weight.

The need for some form of vaccination against tuberculosis will probably always be felt, in spite of the forty to fifty per cent decrease in the death rate in certain educated communities.

Vaccination, applied fully and repeated once or twice in the age of greatest predisposition, has almost abolished smallpox whenever it has been tried. Nothing else has done this, and all localities fail that try to get along on sanitation alone.

The question of applying our procedure to man is to be approached with considerable care. Should he already have tuberculosis in any form it is difficult to imagine that the addition of one bacillus to his probable millions could harm him, and yet even more difficult to imagine it could possibly do him any good. But should that unit begin to produce some immunity, especially a local immunity, the subsequent introduction of say five, and then perhaps multiples of five or less, might find the way prepared for their destruction and so general immunity might progress.

Perhaps the highest art to-day in the treatment of pulmonary tuberculosis consists in the control of the patient's inoculating himself, or autoinoculation, resulting from exercise, elaborated by Paterson and Inman.[35]

Such autoinoculations must result in the discharging into the lymph and blood channels from the foci of disease not only toxins but in all probability live bacilli. It would be of great interest to make a study of blood cultures before and after such exercise. We have ourselves observed in similarly exercised patients changes resulting in their opsonic indices of greater extent than we have found produced by the inoculation of tuberculin. F. Klemperer inoculated tuberculous patients with cultures of virulent bovine bacilli.[36] Moeller inoculated tuberculous patients with a culture of human bacilli, modified by passage through the crocodile.[37] Every one knows that a local suppurative tuberculosis occurring in a consumptive improves his condition and increases his resistance.

We have inoculated five tuberculous men. The inoculations have always been made in the leg, arm, or forearm in such a manner that each new injection was received just below the former. The reason for this being that should any local immunity be established the new bacilli would perish more readily passing through such an area.

Case I. — Catarrhal tuberculosis, involving a large part of the left lung and the apex of the right lung. No expectoration or cough. Frequent clearing of larynx. Moro inunction test negative. Negative from X-ray confirmatory of physical findings.

Dose: One tubercle bacillus, culture (B). Sixty days later no trace whatever of inoculation site.

Case II. — Catarrhal tuberculosis. Both apices. No expectoration or cough. Frequent clearing of larynx. Moro reaction positive. Fluctuating opsonic index to tubercle. Negatives from X-ray confirmatory of physical findings.

Dose: One tubercle bacillus (B) followed by five tubercle bacilli after six days. Sixty days later no evidences of either site of inoculation.

Case III. — Extensive pulmonary tuberculosis. Turban Stadium, III. Persistent fever one year, 99–100° F. Tuberculin treatment three months without results. Sanatorium treatment without results. Patient confined absolutely to rest at home without improvement. First inoculation one tubercle bacillus (Culture B). Subsequent inoculations at intervals of four to seven days; 5, 12, 20, 50, 100, 150, 150, 200, 250, 300, 350, 400, 450, 500, 500, 500. Following the ninth dose the temperature fell to and remained normal, and has been normal ever since. Patient is now up and walking, and comes to the laboratory for inoculation. There has been no reaction of any kind, local or general. Patient is amazed and delighted at his improvement. Tubercle bacilli are still present in the sputum. Physical examinations reveal a very much drier condition of his chest and markedly less sputum is raised. It is unfortunate that the opsonic index was not studied before inoculation. Following the eighth dose it was 1.23; following the tenth 1.44. An inoculation of three hundred tubercle bacilli with index at .75, four days later became 1.04. Subsequent to the inoculation of 350 it has been 1.14; 1; 1.07; 1.13. Still under inoculation. General health excellent; process arrested.

Case IV. — Acute pulmonary tuberculosis. Turban Stadium, II. First inoculation, fifty tubercle bacilli. Subsequent inoculations at intervals of four to seven days, 100, 150, 200, 250, 300, 350, 400, 450, 500, 500 bacilli.

Opsonic index before inoculation was started, .69. Following the dose of 250, .92. Four days later, 1.07. Seven days later, .6.

Subsequent observations have been .83; 1.26; .89; .98; 1.08.

Following the sixth inoculation all fever disappeared and cough was much improved. Weight was being gained. After seven weeks able to do light work.

January, 1909. Discharged cured after twelve further inoculations of five hundred bacilli.

Case V. — Dr. G. W. P. Extensive pulmonary tuberculosis. Ten years' duration. Turban Stadium, III.

Tuberculin treatment two years, sanatorium three years, without distinct benefit.

First inoculation, one tubercle bacillus. Subsequent inoculations at intervals of four to seven days, 5, 25, 125, 250, 250, 475, 500, 500, 500, 500, 500 bacilli.

The opsonic index at the beginning was .87. Subsequent observations after four and seven days subsequent to inoculation have been 1; .72; .9; 1; 1.05; 1.04; .88; .91; 1.07: 1.28.

Estimation of the degree of lysis according to the lytic index[41] was three times normal, following the first dose of 500.

Patient feels generally better, although he has once or twice experienced a feeling of languor following inoculations. General improvement cannot be demonstrated, although the doctor feels a decided general gain. No apparent harm has been done. There have been no reactions, local or general, and no lesions of any character at inoculation sites.

The chief interest of these cases lies in the fact that no harm has been done that can be recognized, after one hundred and fifty days (Jan. 9, 1909); that remarkable benefit accrued in two cases considered hopeless, and this, with so few bacteria injected in proportion to what would be present in tuberculin therapy. From our work on guinea-pigs we feel that some immunity can be safely produced by beginning with the subcutaneous inoculation of one tubercle bacillus, and we regard such a method as being harmless, and more than probably being beneficial to an already tuberculous man.

[Our grateful acknowledgment is due to Dr. G. W. Morse of Colorado Springs, and Mr. Clarence W. Lieb, B.A., of Colorado College, or a great amount of painstaking laboratory work connected with these experiments.]

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ON THE LARGE WHITE OR SOAPY KIDNEY.*

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Frerichs in 1851, from his observations on the kidneys, noted that "in general the fat content of the kidney in Bright's disease is less than what one would expect from the microscopic appearance, and one must not consider all the droplets in the epithelial cells as fat."

Many have drawn attention to the fact that what appear to be fat droplets in the epithelial cells of the renal cortex are not really such, and do not give the reaction for the neutral fats of the higher series.

In the careful analyses of Rosenfeld and also of Orgler, they independently came to the conclusion that the apparently fatty degeneration of the kidney as it is seen in the large white kidney, does not contain more, but actually less fat than normal. Stoerck comes to similar conclusions, and declares that the constituent of the cortical cells giving them their white color is protagon.

Various are the reports concerning crystalline bodies found in the kidney, but little information can be gained from those described from microscopical sections alone. Much of the confusion concerning these bodies has developed from the varied interpretations of the bodies and their microchemical reactions. Globular lipoid bodies have been jumbled together, into the class of myelins, other crystalline bodies are listed under protagons, while still a third variety of fine acicular crystals have remained undefined. I am here referring only to substances within the epithelial cells of the cortex or lying in the interstitial tissue.

In 1854 Virchow noted in the scrapings of diseased lungs and in the expressed mucus, peculiar round and transparent bodies which under the ordinary microscope exhibited a double contour. Bodies of the same nature he also observed

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* Received for publication Nov. 2, 1908.

in the spleen, thyroid, egg-yolk, testicle, ovary, pus and bile. His attention was still further attracted to this substance in finding that its reaction to simple reagents showed a similarity to that of the sheath of medullated nerves. To the substances in general he gave the name, "Markstoff" or Myelin.

Myelin, in Virchow's description, was a substance occurring often in globular form, but which on the addition of water swelled up and assumed various shapes with double contours. The substance he reported was readily soluble in hot alcohol, ether, chloroform, and turpentine.

Previous to Virchow, Henle had noted these peculiar bodies in the sputum and considered that they were fat, while Funke and Meissner had described similar structures and referred to them as corpora amylacea.

Liebreich undertook a chemical, or rather a microchemical analysis of the substance in question, and came to the conclusion that myelin was a mixture of the decomposition products of protagon with protagon itself. Diakanow, on the other hand, held that myelin was a chemical combination of protagon with lecithin, and he reported that the chemical reactions of such a combination were identical with those of myelin.

Following this several more accurate chemical analyses were made of brain and nervous tissue, isolating therein lecithin, neurin, cerebrin, protagon, cholesterin, and others. Many of these substances have been reported as being associated with the constitution of myelin, but these mixtures and compounds do not correspond to all the types of substance which Virchow designated as myelin. No definite or distinctive chemical reaction has been found to differentiate myelin from other substances, although many chemical properties have been determined — which when taken together constitute an equation signifying myelin.

Beneke examined materials showing myelin bodies, in various organs, and he noted that the substance was to be found in processes of degeneration where cholesterin was present. From this he concluded that, without cholesterin, myelin bodies do not occur. This was, however, demonstrated not to be the case. Neubauer and also Kohler found that myelin forms could be developed outside the body by allowing oleic acid and ammonia to run together. The substance, an ammonium oleate, had the microscopic properties of myelin as it is seen in the tissues, and they concluded that myelin was not a definite chemical body, but that it might be a substance of varying composition. This conclusion was based on the fact that besides the ammonium oleate a combination of oleic acid with other bases produced similar results.

Panizza denied the occurrence of myelin as a chemical entity and believed that the presence of a semifluid mass (dickfluessige Masse) in a liquid showed bodies similar to myelin forms. In short, he considered that myelin was only a physical state. He further stated that myelin occurring in the sputum was mucin, and was developed in the epithelium of the respiratory tract.

It is evident that Panizza based his studies upon the microscopical appearance of certain droplets within and outside of the cells. Certain known chemical reactions were not at his command and from the unstained microscopical appearances he has confused bodies of different chemical composition.

Small globules of refractile substances, having the property of swelling in water and showing a double contour at their periphery are as Panizza demonstrated of different kinds — but they are not all myelin. We do not wish to say that myelins are or must be of the same chemical composition, but they must be differentiated from mucins, fats, and certain other lipoid bodies.

Myelins are, under certain physical conditions, anisotropic, and when in this state they show peculiar figures by which they may be recognized. As, however, these same myelins may alter their optical characters and appear as isotropic granules or needle-shaped crystals, the polariscope alone cannot be depended upon to differentiate them from other substances. Nevertheless, the polariscope offers a ready method of determining in fresh and wet organs the presence of some of the myelins, and up to the present no other substances in the animal body have been determined which exhibit the same figures and optical characters as exhibited by the myelins. We must not, however, lose sight of the fact that other definite chemical bodies have been shown to possess the property of anisotropism and the stage of fluid crystals, and it may yet be shown that bodies of various kinds possessing these properties exist in the animal body. One such — protagon — I shall discuss later.

For the more extensive literature on the subject I refer the reader to the article by Adami and Aschoff. In their studies they observed that quite a variety of known chemical substances yield myelin forms of the anisotropic variety. Thus sodium, potassium and ammonium oleate; ammonium palmitate and stearate; cholesteryl oleate, palmitate, stearate and butyrate; a mixture of lecithin and cholesterin; cholin oleate; and mixtures of lecithin with ammonia, potassium hydrate or sodium hydrate, will all afford myelin forms of the anisotropic type. These authors were the first to point out that of these different chemical substances only those can be taken into consideration (as regards the myelins of the human system) which will retain their anisotropic myelin type at body, or at least room temperature. On this argument, the compounds of stearic and palmitic acids may be left out of consideration, and the only ones which may be considered are certain oleic acid compounds. Of these compounds they believe that cholesteryl oleate and cholin oleate play the most important part.

It is important to point out that Adami and Aschoff observed the presence of spheroliths as well as the fluid spherocrystals. The former were of two kinds, that of solid spheres and that of spherical masses of closely packed radiating acicular crystals, which might readily be distinguished from anisotropic fluid crystals in that they lacked the oily consistence of the latter. As I shall point out later, I was able to demonstrate that the anisotropic myelin forms (fluid crystals) may pass over into the anisotropic spheroliths with radiating needle-like crystals, in the process of dehydration. Adami and Aschoff were able to produce similar results by the action of formalin on cholin oleate.

Quite similar observations were made by Loehlein and later by White. They found that frozen sections of formalin-preserved tumors showed the presence of fine acicular crystals which when warmed became converted into globules showing anisotropism.

Kaiserling and Orgler and others have found that anisotropic globules are normal constituents of the adrenal. White notes that the crystals and globules in the tissues in some cases can be shown to contain cholesterol and a fatty acid, which becomes separated on warming. In other cases the globules show no evidence of fatty acid. The presence of the cholesterol in these globules was indicated only by the brown color in these globules when the tissues were treated by eighty-five per cent of sulphuric acid, while the fatty acid was demonstrated by staining a section with nile blue, and obtaining a blue color in the globules. A brown discoloration of the tissues and fluids is almost constant in treating sections with eighty-five per cent of sulphuric acid, and this I have found is an uncertain test for cholesterol in tissues. The blue coloration obtained in staining sections with nile blue is not to be depended upon as indicating in every instance the presence of fatty acids, as the shade of the color is influenced by external circumstances.

Although this author has demonstrated the physical properties and variations of many chemical substances, and has shown that artificial myelin forms owe their characters to water, "acids" or "alcohols of crystallization," the relationship of these substances to those in the tissues is only made by analogy. No analysis or isolation of the anisotropic globules or myelin forms as they occur in the tissues is made.

The following investigation concerns only the so-called myelin bodies found in the kidney. By myelin forms I refer to those substances which appear as spherical or varied shaped bodies, having under the microscope a double contour at the borders, which have the property of swelling up when in contact with water, are soluble in alcohol, and which are or can be made to appear anisotropic under the crossed Nichols prisms.

The chemical reactions of the "myelins" as I have found them in the kidney I shall give below. As the identity of the myelins from the various organs has not been determined, the reactions here given and the inferences drawn do not apply to myelins derived from other organs. I am of the opinion that there are myelins of various kinds, which has led to the confusion now existing concerning their general reactions.

It was my endeavor to obtain the myelin bodies in as pure a state as possible, and to control each step in the work the various extracts were examined with the polarizing microscope.

MATERIAL. — The kidneys were obtained from cases suffering from chronic nephritis. The organs were of the large white variety and were typical examples of chronic parenchymatous nephritis. The cortex was thick and of a glistening white color, giving the impression of extensive fatty degeneration. The dark medulla contrasted sharply with the pale cortex. There was no outward evidence of chronic interstitial disease, though this was apparent in the microscopic sections.

The amount of fat that could be demonstrated in frozen sections stained with Sudan III. was quite the reverse to what was expected from the macroscopic appearance. Instead of finding extensive fatty deposits in the kidney tubules, little or no fat was present in the epithelial cells of the cortex or medulla. Only a few cells of fatty infiltration were present about the larger blood vessels. The white and fatty appearance seemed difficult to explain. Scrapings from the cortex examined under the polarizing microscope showed a great number of doubly refractile bodies, and this number was readily increased by warming the slide until dry and then exposing the smeared surface to flowing steam. In all appearances the kidneys were similar to those so extensively described by Stoerck.

In these kidney scrapings the anisotropic bodies were spherical and varied in size from two to ten microns. The appearance was the same in all, there being a dark cross placed on the illuminated sphere. If allowed to dry, the bodies kept their form, but the refractile portions of the bodies gradually showed the development of crystals, radiating from the center of the sphere. These radiating crystals were individually composed of needle-shaped crystals which, as the specimen became dehydrated, became massed together forming single, double, or quadruple sheaves. If these desiccated crystals were exposed to flowing steam for a few minutes or longer, they again assumed their original form of anisotropic globules.

The fresh kidney scrapings were treated with various reagents, and the results watched under the microscope. (1) When treated with absolute, ninety-five per cent, and eighty-five per cent alcohol the doubly refractile bodies disappeared, or at least lost their doubly refractile nature. On the evaporation of the alcohol amorphous isotropic granules appeared, which, however, on the addition of moisture and heat took on the characters of the anisotropic globules, save that now these globules were much increased in size, up to 20 μ. (2) When the scrapings were treated with neutral ether, the greater number of the doubly refractile globules lost their anisotropism, and on the evaporation of the ether, needle-shaped crystals were left behind in their stead. These acicular crystals were distributed in small masses or singly through the field. The needles were fairly long and tapered to fairly sharp points. The ether had another effect on the anisotropic globules when these were collected in masses. The addition of ether caused these globules to become stretched out into myelin forms with anisotropic ends showing the dark cross. These long myelin forms were quite similar to the appearance of those seen in brain tissue, or the " myelin-forms " produced by bringing semifluid sodium oleate in contact with ether. (3) When treated with excess water the anisotropic globules disappeared, but reappeared again on slow evaporation, or if heated to desiccation would reappear by exposure to steam.

Too much weight must not be placed on the microscopic absence or disappearance of these bodies, when treated with certain reagents, as denoting true solution. It is quite possible and even probable that the globules lose their anisotropic nature when in contact with certain substances, while, however, they do not become dissolved in the substance. It is most likely that the disappearance of the anisotropism of the globules when in contact with ether is of this nature. Frozen sections of some of the kidney tissue preserved in formalin did not show any of the anisotropic globules seen in the fresh scrapings, but in their stead acicular crystals were scattered through the organ, some appearing within vacuoles in the epithelial cells, others in the interstitial tissue.

At autopsy the greater portion of the kidney substance was freed from fat and the loose tissue of the capsule and pelvis. The kidney substance was then placed in a clean jar of neutral ether and allowed to stand for five days. At the end of this time the ether showed some absorption of the neutral fats. The aqueous juices of the kidney became expressed and formed a small quantity of brown fluid at the bottom of the jar.

On evaporating some of the filtered ethereal extract and exposing the dried slide to steam, the anisotropic globules were present in small numbers. Some fats and other extractives were also present in the evaporated residue.

Some of the watery layer at the bottom of the ether extract was collected, and slowly dried. This dried material showed innumerable anisotropic globules mixed with a pigment and débris. The addition of a little water to convert the residue into a pasty mass brought to light a great many more of the anisotropic globules. Occasional cylindrical myelin forms were present among the globules, and it was noted that the anisotropic globules could be changed to the cylindrical myelin forms by pressure on or dragging of the coverslip.

About one-fourth of one kidney was taken from the ether and ground up in a mortar. The ground material was then extracted with absolute alcohol (in excess), in the cold. The flask containing the mixture was frequently shaken and kept for five days at room temperature. The alcohol was then filtered clear. A small amount of the alcohol was evaporated, and the greasy looking residue was moistened with water and warmed. Many anisotropic globules appeared in the specimen. The globules were larger than those found in the original kidney scrapings.

A small quantity (one cubic centimeter) of the alcoholic filtrate was treated with chloroform in excess (ten cubic centimeters). A heavy white milkiness resulted which, when the fluid was allowed to stand, formed a scum at the top of the fluid. This scum under the microscope was made up of needle-shaped crystals, which had no definite arrangement to each other. It was found that if the chloroform alcohol mixture were evaporated as soon as the precipitate appeared no needle crystals were to be found in the fluid, but instead isotropic granules similar to those seen in the original kidney scrapings when treated with alcohol. Both the acicular crystals and the isotropic granules could be changed into anisotropic granules by moistening them with water and warming, or exposing them to warm steam.

The entire alcoholic filtrate was then precipitated with excess chloroform, and the resulting white precipitate was filtered out and washed with fresh chloroform. This residue on the filter was then dissolved in new absolute alcohol.

From the last alcoholic solution the various types (anisotropic globules, needle-shaped crystals, and isotropic granules) of residue could be obtained and each could be changed into the other form by heat, water, or chloroform.

When some of the reprecipitated (chloroform and alcohol) residue is treated with ether no solution of these crystals takes place. In the presence of ether the crystals arrange themselves radially or in double or quadruple sheaves. The radial masses are made up of fine needle-shaped crystals which under the polarizing microscope are isotropic. The arrangement of the crystals is quite like that of phenylglucosazone. When the ether is evaporated from the undissolved crystals, the latter can be converted into the anisotropic globules having the maltese-cross dark areas.

We are able, therefore, to obtain a recrystallized product from the alcoholic extract which contains anisotropic globules similar to those seen in the original kidney scrapings. From the reaction of ether on the isolated myelin bodies in their crystallized form, we must uphold the insolubility of the anhydrous form in ether. This is further proved in the following experiment. When some of the alcoholic extract was mixed with an excess quantity of neutral ether an opalescence immediately appeared, and a white flocculent precipitate resulted. A few doubly refractile globules were present in this precipitate which consisted mainly of amorphous isotropic granules. In the filtrate no anisotropic bodies could be demonstrated. That the globular anisotropic form of the substance disappears, to a great extent, in apparent solution when in contact with ether on a slide is to be demonstrated in each step of the experiments, but this conversion of the globular type of myelins into the long cylindrical type, the isotropic amorphous form or the crystalline product appears to be rather a physical change and not solution, and I would refer the reader to a paper on an analogous subject by Bayliss and Starling.

Anisotropic globules obtained on a slide by the evaporation of the alcoholic extract, gradually disappear when brought into contact with a two per cent solution of osmic acid. The gradual dissolution of these globules can be observed under the polariscope — and concurrently may be noted the blackening of the fluid. Differing from solution of the common oleates the blackening does not take place immediately, but requires some three to six hours for its completion. In making these reactions with osmic acid, and using fair quantities of the residue in a watchglass, the resulting color was always black and not the gray black spoken of by Kaiserling and Orgler. Nor was I able, after the myelin substances had become thoroughly blackened, to remove the color by the use of xylol or chloroform.

If the reaction with osmic acid be carried out in a solution, the fluid becomes blackened, and a black granular precipitate sinks to the bottom. This precipitate did not take any particular crystalline form.

For comparison, a pure sodium oleate was prepared. The pasty mass showed innumerable anisotropic globules and myelin forms. The addition of osmic acid to this preparation led to a gradual disappearance of the anisotropic globules, so that nothing could be seen of them. After a few minutes the solution became brown and then in a short time turned black. The dissolution and disappearance of the anisotropic globules was quite similar to that observed in the kidney extract. A black and structureless precipitate also developed in the test fluid, and anisotropic globules could not be obtained from the osmic acid mixture.

The similarity of the anisotropic globules in their reactions with perosmic acid to the globules present in sodium oleate paste was striking — the difference being in the length of time in which the blackening took place.

Tests for cholesterin carried out on the alcoholic solution, or on the crystalline precipitates therefrom, proved negative. Neither the Liebermann-Buchard nor the Neuberg-Rauchwegers reaction demonstrated the presence of this substance in the extracts, nor were cholesterin crystals ever encountered in the microscopic examination.

By accident a watchglass with some dried chloroform-alcoholic precipitate became immersed in boiling water. On removal from the water the granular precipitate had become converted into a gelatinous pasty mass. Anisotropic globules were not present in this mass.

Some of the alcoholic extract was treated with alcoholic solution of calcium chloride. This led to a marked opalescence with a succeeding flaky white precipitate. Warming the mixture hastened the formation of the precipitate. The addition of lead acetate to the watery extract led to a heavy white precipitate without the appearances of opalescence.

The anisotropic bodies when obtained on a slide from any of the previous cases and treated with two per cent acetic acid did not alter their shape or appearance.

When a concentrated sulphuric acid or a mixture of concentrated sulphuric acid and formalin (Goloditz' reaction) was brought in contact with the anisotropic globules under the microscope these globules disappeared as if in solution and no trace of them remained. There was no brown or black staining of the globules or the solution to indicate cholesterin. During the process, while the sulphuric acid was acting on the anisotropic globules, very fine particles were given off. These particles may have been fine gas bubbles, but this was not definitely determined.

When the anisotropic myelin globules are treated with fifteen per cent hydrochloric acid the globules quickly lose their anisotropism and disappear.

When the myelins (in the anisotropic form) have been isolated in a pure state and allowed to dry they are not dissolved in chloroform or ether. This apparent want of conformity with Virchow's postulates we refer elsewhere.

It has been pointed out by many that the admixture of oleic acid with cholesterin yields anisotropic globules which may take the type of the cylindrical myelin bodies. I have prepared such a mixture and can verify the observations of Adami and Aschoff and others. Such a mixture is soluble in alcohol, but the reactions of this solution differ from those of our alcoholic extract of the kidney. When treated with chloroform or ether, the alcoholic solution of the cholesterin oleic acid mixture remains unchanged and no precipitate is obtained by an excess of the reagents. In these solutions the cholesterin can readily be detected by dry or wet tests.

FATTY ACIDS. — A chemical examination was undertaken to determine the nature of the substance we were dealing with, and proof conclusive was obtained that fatty acids were present. Some of the alcoholic extract was precipitated with chloroform, and the precipitate was redissolved in absolute alcohol. To this alcoholic solution was added a one per cent hydrochloric acid solution until the whole was decidedly acid. This mixture was thoroughly shaken and after twenty-four hours was shaken out with neutral ether. The ether was then separated and given several washings with distilled water until no more hydrochloric acid could be detected in the wash-water. The ethereal solution was then tested as follows:

(*a.*) Several drops of the ether, when placed on glazed paper, left a permanent fatty stain.

(*b.*) When a small quantity of the ether was allowed to evaporate on a watchglass, clusters of isotropic acicular crystals appeared, which did not take any special arrangement to each other. Some oily globules were also present at room temperature. The acicular crystals melted when warmed at 50° C. and recrystallized at 49° C.

(*c.*) A faintly alkaline alcoholic solution of phenolphthalein became decolorized when treated with a small quantity of the ether residue.

(*d.*) Heating a small amount of the ether residue (by evaporation) in contact with acid potassium sulphate did not give the odor of acrolein.

(*e.*) When some of the ether residue was treated in the cold with a five per cent solution of potassium hydroxide and,

(1) A solution of calcium chloride was added to it, a flocculent white precipitate resulted.

(2) A solution of lead acetate was added to (*e*) a heavy precipitate resulted. This precipitate was partly soluble in ether.

(3) When dried, and the residue was dissolved in alcohol, a flocculent white precipitate resulted when the solution was acidified with a one per cent solution of hydrochloric acid, which precipitate was soluble in ether.

(4) When the potassium hydroxide mixture was slowly evaporated and warmed slightly, there appeared myriads of small anisotropic globules, similar to those noted in the early experiments and kidney scrapings, and showing the characteristic dark cross.

A quantity of the material was precipitated with lead acetate similar to that obtained in reaction (2). This precipitate was filtered, washed with distilled water and allowed to dry. This precipitate was now treated with ether and thoroughly extracted. The greater portion of the precipitate was found to have dissolved in the ether. The ether was again filtered and the filtrate allowed to evaporate. The residue from the ether evaporation was collected and suspended in distilled water and acidified with weak hydrochloric acid solution. The mixture was then shaken out with ether, and the ethereal extract separated. When this ethereal extract was evaporated small transparent globules remained on the watchglass. These globules were of an oily consistence at room temperature, but when placed on a salt and ice mixture became crystalline. The globules were soluble in alcohol, ether, and chloroform. When treated with a five per cent potassium hydroxide solution anisotropic globules were obtained, and the whole was soluble in water. The lead precipitate, which was not soluble in ether, was treated with a weak hydrochloric acid solution and the mixture shaken out with ether. On the evaporation of the ether a very slight amount of residue was obtained, which was made up of rather coarse isotropic crystals, arranged in small masses which, when heated, would melt at 61° C. These crystals were soluble in alcohol, ether, and chloroform, but were insoluble in water. When treated with a five per cent solution of potassium hydroxide and calcium chloride solution added, a white precipitate results. The material, however, was in too small a quantity to determine whether it was composed of one or more substances. Clear it was, though, that this substance belonged to the higher fatty acids. (The melting point of these crystals was, I believe, influenced by incomplete dissociation from oleic acid.)

That these fatty acids were in combination in our original alcoholic extract with a base or substances acting as bases, is clear from the behavior towards chloroform and ether, and the subsequent reactions.

The fatty acids which we have isolated, and which have been shown to form soaps in the presence of alkalis, occurred in a mixture, there being a portion which at room temperature appeared in a crystalline state, and another larger portion which was present in small oily globules. The latter, which had the properties of being in an oily state at room temperature, crystalline at 0° C., forming soluble salts with sodium and potassium, and whose lead salt was soluble in ether, was clearly demonstrated to be oleic acid.

With the small amount of material at our disposal, I was not able to determine with certainty the nature of the other fatty acids which were liberated.

BASES. — A determination of the bases separated from the fatty acids was also made. Calcium and magnesium were not present. On the other hand, sodium and potassium were found in fair quantities. (The various reagents used during the experiments were also examined for these substances with a negative result.) Proteid matter either free or in combination was not demonstrable.

A. Schmidt in 1897 determined chemically that myelins of the sputum were "protagon," and that these were normal secretions of the bronchial and tracheal mucosa. Besides protagon he also found small amounts of cholesterin and lecithin in these secretions.

In 1906 Stoerck published a most interesting research concerning the "protagons" of the large white kidney. In this organ he found quantities of "protagon" in the epithelial cells, as well as in the interstitial tissue, while occasionally the substance found its way into the lumina of the tubules.

His examinations were mostly of a histological character and the determination of the protagon was confined to frozen sections of fresh and formalized tissue. The appearance of the protagon in the latter sections was quite similar to the needle-shaped crystals which I found in the formalin specimens examined, while in the fresh tissue he encountered the anisotropic globules as we have described them above.

Stoerck found that these doubly refractile globules take the Sudan stain but faintly, and are soluble in alcohol, acetone, ether, benzine, chloroform, and xylol. I must again point out here that Stoerck determined the solubility of the substance under the microscope, and that rather than demonstrating that the anisotropic globules were soluble in the above-named substances he proved that they altered their optical characters — in some cases on account of solution. He found that these optically active substances were to be found normally in the adrenal, and are at times to be seen in tumors and in atheroma.

Panzer, who carried out the chemical analysis for Stoerck, found that the protagon which Stoerck was dealing with differed from the protagon of the brain, in that it was free from nitrogen and phosphorus. For true protagon (so-called), nitrogen and phosphorus are essential constituents.

It would seem to me that the substance Stoerck was dealing with is identical with the myelins referred to in this paper. There is at least great similarity in his histological findings and mine. On the other hand, we disagree in the matter of solvents, which I believe is due to the fact that Stoerck relied on the change in the (microscopic) optical activity as denoting solution.

Concerning the occurrence of "protagon" in the kidney Stoerck believes that it denotes a process of degeneration.

Panzer later made a more detailed study of the so-called protagon of the kidney. He isolated a substance by precipitation from cold acetone, and demonstrated that the crystals so obtained had anisotropic characters similar to the original globules in the kidney. He does not state, however, by what method the crystals were altered to the anisotropic globules. The crystals were found to be a compound of oleic and another fatty acid with cholesterin.

In Panzer's determinations, however, the possibility of the cholesterin remaining in the solution as a mixture cannot be ruled out, and it may be that this substance was not united with the fatty acids.

In a recent paper White has reported the occurrence of needle-shaped crystals in various tissues. For several of these he determined the melting point and by analogy of their characters has concluded their chemical composition.

It need hardly be mentioned that in the fresh kidney tissue crystals of various kinds are to be met with (tyrosin, urates, phosphates, "protagon" and others) while in the formalized tissue the varieties of crystals are further increased. The action of the constituents of the commercial formalin, containing formic and some acetic acid on the tissues, must not be forgotten. Hence, the deductions based on the crystalline bodies present in tissues preserved in this medium must be made with care.

The melting point of mixtures of fat or fatty acid crystals gives us no clue as to the constituents. It is only after the material is gained in its pure state that this test serves us a purpose. I can therefore not claim to have determined the exact nature of the second fatty acid isolated.

CONCLUSIONS.

To sum up, we have found that the large white kidney contains a compound, myelin, so-called, soluble in alcohol, from which it can be precipitated by chloroform. This precipitate with chloroform possesses the optical characters of the substance seen in the fresh organ. On analysis this substance contains fatty acids (oleic and other fatty acids being determined). These fatty acids were in combination with sodium and potassium, while neither proteids nor cholesterin could be demonstrated in the compound. The fatty acids when isolated, and again reunited with sodium and potassium, gave anisotropic globules similar to the original material.

It is to be pointed out that the large white kidney which we have usually considered as the fatty kidney, is in reality a soapy kidney and that part at least of the myelins contained therein consist of soaps of oleic acid. These fatty compounds are not readily demonstrated by the ordinary staining with Sudan III., but can be obtained by extraction with alcohol.

[I am greatly indebted to Professor Adami for the many suggestions he has given me in this work, and to Professor Aschoff for the kind assistance given me during my stay at his laboratories in Freiburg, Germany. I wish to thank Dr. Bruere for the kind assistance he has given me in making the chemical analyses.]

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THE ORIGIN AND DEVELOPMENT OF FOREIGN BODY GIANT CELLS.*

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Although foreign body giant cells are of common occurrence, there is little said in text-books about their origin. Ribbert thinks that the fixed connective tissue cells are their chief source, and mentions endothelium of blood and lymph vessels as playing a part, but as being of less importance. He further says that these giant cells arise from ordinary cells in two ways, — through the enlargement of a single cell and through the union of several.

The method adopted in these experiments for the study of their origin was the injection of agar agar subcutaneously in rabbits. The jelly, as prepared for cultures, was broken into small masses with a sterile needle and injected with a sterile hypodermic syringe. Three animals were used. The sites of injection in the first were not rendered aseptic. In the case of the other two, the skin was washed carefully with seventy per cent alcohol. The injections were made at such intervals of time that when the animals were killed the ages of the injections covered a considerable range. In the first animal the periods during which the injections had been in the tissues were as follows: eighteen hours, one, two, three, four, five, eight, ten days, and in two-day intervals to twenty-two days. In the second rabbit the intervals were longer, and the oldest injection was of forty-four days' standing. To the six and the twenty-six-day injections lamp black was added in order to differentiate more clearly the phagocytic cells. For this purpose the agar was melted and lamp black was stirred in, after which the agar was allowed to cool before being broken up and injected. A study of the material from the first two rabbits revealed a need of injections between five and eight days, and it also showed the

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* Received for publication Dec. 6, 1908.

need of making the injections smaller, for the mechanical irritation had caused an inflammatory reaction which tended to confuse the problem. Injections were made in a third rabbit every day for ten days, and the rabbit was killed immediately after the tenth injection. In addition to the subcutaneous injections, intramuscular injections were made in this animal on the fourth and fifth days before death. In the first two rabbits the injections amounted to between one and two cubic centimeters each; in the third rabbit the amount was reduced to between .2 and .5 cubic centimeter.

It was hoped by means of phosphotungstic acid hematoxylin to stain the fibroglia fibrils of the connective tissue cells so that they would stand out in sharp contrast to the endothelial cells. For the most part this was found to be impossible for two reasons: first, the connective tissue cells in the rabbit are smaller than in man, and the fibroglia fibrils are very delicate, so that in many preparations the fixative did not penetrate quickly enough to preserve them. In a few lesions, however, they could be demonstrated distinctly. Second, fibrin often forms a network around the endothelial cells and stains by the same method, so that the picture afforded by them is sometimes confusing.

Three slides were prepared from the lesion produced by each injection, one stained with eosin and methylene blue, another with phosphotungstic acid hematoxylin, and a third with the aniline blue connective tissue stain. The sections stained with eosin and methylene blue were studied the most, but frequent reference was made to the corresponding slides stained by the other methods.

Sections from the injection made a few minutes before death show a quantity of agar in the subcutaneous connective tissue which has been torn to make room for it. Loose ends of collagen fibers extend from the edge of the lesion among the agar masses, and a few detached shreds are seen throughout the injection.

The reaction which appears in subsequent stages may be divided into exudative and proliferative. The earliest exudation is serum which permeates the entire injection, as is shown by the deposit of fine particles which may be either fibrin or an albuminous precipitate due to fixation. This is best shown by the aniline blue stain, for with this the fresh agar stains pale blue and is almost transparent, while the deposit stains red. At eighteen hours the particles are distributed so uniformly that the whole mass appears reddish and homogeneous with low power. This condition persists in the larger masses in the oldest injections studied. In the smaller masses which have been surrounded by giant cells, the particles have apparently been removed, leaving the agar clear blue as in the fresh injection, while in some cases there are dense deposits of particles at the boundaries between the remaining agar and the cytoplasm of the giant cells.

The next exudative change is an extensive invasion of the injection mass and the adjacent tissue spaces by polynuclear leucocytes. This invasion reaches its maximum at twenty-four hours, while at two days those leucocytes which have penetrated farthest into the agar have begun to disintegrate. The disintegration becomes more extensive and in the later stages few living cells are left, while a good deal of nuclear detritus is present. Of these leucocytes the neutrophiles are at first in a very large majority, but the proportion of eosinophiles increases till at the ninth day in Rabbit 3 they are about as numerous as the neutrophiles. The polynuclear cells are more abundant in Rabbit 1 than in Rabbit 3, the injury to the tissues being greater in the former.

Lymphocytes are abundant in Rabbit 1 from twenty-four hours to sixteen days or later, but especially at four and five days. In Rabbit 3, where the injections are smaller, they are very scarce. They take no part in giant cell formation.

One more type of cell appears in the exudation. This is a mononuclear cell whose nucleus is irregular in shape, about the size of an endothelial nucleus in a vessel wall, and contains less chromatin than that of a lymphocyte. The cells of this type are abundant in Rabbit 3 at twenty-four hours, both inside the blood vessels and outside of them close to the agar. They may also be seen migrating through the walls of the blood vessels. Several instances of this were found in Rabbit 3 at two days (Figs. 1, 2 and 3). The cytoplasm of these cells at twenty-four hours is scanty and stains faintly bluish with the eosin and methylene blue method. At two days these cells are more abundant and their appearance is much the same except for a slight increase in the amount of cytoplasm. At three days there is a great increase in their number. For the most part their appearance is the same as at two days, but in many there is marked hypertrophy. The cytoplasm has increased in quantity and takes a deeper stain of a predominantly bluish tint; the nuclei are much the same, although in some cases they are larger and rounder, and their chromatin appears in denser masses with more clear space between. Many cells are found at stages intermediate between the extremes. After these changes, their appearance is typical of the large mononuclear leucocytes of endothelial origin. They are undoubtedly identical with the latter. At three days the mononuclear cells have almost wholly replaced the polynuclear leucocytes at the surface of the agar where at one day the latter were predominant. The further increase of these cells will be considered under proliferation.

At twenty-four hours there is first seen a growth of young blood vessels like that which occurs in granulation tissue. It is more marked at two days, and reaches its maximum at about five days. There are many mitotic figures in the endothelium of the vessel walls at two and three days. Hypertrophy of the endothelium in the preëxisting vessels is also conspicuous, and many of the cells undergo mitosis (Fig. 4). The nuclei are enlarged and the arrangement of the chromatin has undergone a change similar to that noted in the free mononuclear cells, the cytoplasm has increased in bulk more than the nuclei and stains distinctly blue with eosin and methylene blue.

In the tissue spaces around the agar the number of free endothelial cells shows a marked increase over the normal at twenty-four hours in Rabbit 3, and a further increase up to nine days, when the animal was killed; the maximum is almost reached at five days. At two days mitoses are present in these cells and their abundance shows a similar increase throughout the series (Figs. 5, 6, 7, and 8). In one instance, at seven days, a multiple mitosis was found.

Whether these rapidly multiplying cells are derived mostly from those which existed in the tissue spaces before the lesion or mostly from those which emigrate through the vessel walls, it is impossible to ascertain. Since, however, the injury causes the same hypertrophy and proliferation both in the fixed endothelium and in the free endothelial cells, it seems probable that the free endothelial cells from both sources are influenced alike. It is also possible that some of the young vessels break up and that the cells of the endothelium become free, thus adding to the number; but it is more probable that these vessels atrophy later as they do in granulation tissue.

A fact which tends to confirm the assumption that the mononuclear cells about the agar are endothelial is that the changes which their nuclei and cytoplasm undergo from the first to the third and fourth days are so closely paralleled at the same stages of the reaction in the fixed endothelium of the vessels. The phagocytic property of the mononuclear cells about the agar was demonstrated by the lamp black injections where they were found to be filled with ingested particles of carbon, while lymphocytes and plasma cells close beside them had taken up none.

This brings us to the formation of giant cells. It has already been stated that in Rabbit 3 at twenty-four hours many mononuclear cells are found close to the agar, and that at three days their number is greatly increased. Some of these cells are scattered about in the tissue spaces, but most of them are in contact with the agar and in many cases no sharp line can be seen dividing the cytoplasm from it. At three days much of the agar is lined with these cells and small masses are completely surrounded by them. At four days this condition is found more extensive (Fig. 9), in many cases the cells are so crowded together that there is only a narrow crack between them (Figs. 8, 10, and

11) and in some cases the cells have coalesced and formed giant cells. Occasional giant cells are seen with as many as eight or ten nuclei in a single section through them. At five days more cells have migrated to the surface of the agar, and there is a great increase in the number of giant cells and in the size of the larger ones. From this time on, the number of giant cells constantly increases. In the older lesions all stages of the process of their formation are found, mitosis in single cells and groups of coalescing cells occurring in the neighbórhood of large giant cells as late as twenty days in Rabbit 1 (Fig. 8). In Rabbit 1 where the injections were larger and the injury to the tissues greater, no giant cells were formed till after five days, whereas in Rabbit 3 they appeared on the fourth day.

The nuclear changes occurring with the hypertrophy of the endothelial cells become even more marked when coalescence occurs so that most of the nuclei of the giant cells are well rounded and the chromatin appears mostly in a single solid mass near the center. The cytoplasm of the giant cells stains blue with eosin and methylene blue like that of the young proliferating endothelial cells and is thus shown to be distinct from the red staining ingested agar, although there is not always a sharp line visible between the two.

The giant cells in a number of sections were searched for mitotic figures and none were found; moreover, in only a few cases were there mitoses in cells actually in contact with the agar. No evidence of direct division was found. It appears, therefore, that nuclear division ceases when fusion begins.

The older lesions in the first two animals show that after about eighteen days the entire injection is surrounded by a solid wall of giant cells and single endothelial cells packed close together. The giant cells have ingested small masses of agar along the surface, while the masses in the center of the injection have not been attacked. A few endothelial cells have penetrated to the masses within the giant cell wall, but they are not in dense clusters such as appear during the active process of giant cell formation.

In the lamp black injections both the single cells which line the agar and the giant cells are full of ingested carbon, and in both cases the carbon occurs almost wholly in the cytoplasm, the agar being nearly free from it. In the larger masses of agar, which have not been surrounded by giant cells, the carbon particles are not evenly distributed, but occur in clusters. It may be that the apparent withdrawal of the carbon from the agar is really due to a migration of the phagocytic cells to the points where the carbon is thickest.

At about five days there appears outside the layer of forming giant cells a growth of connective tissue with young collagen fibers, as distinctly shown by the aniline blue stain. This connective tissue increases and penetrates into the interstices between the giant cells till in the latter stages there is an almost continuous capsule of young connective tissue between the giant cells and the surrounding tissues, and newly formed collagen fibers extend in places through the whole of the giant cell layer. In the later stages of the lesions a few delicate fibroglia fibrils were found in this connective tissue capsule.

SUMMARY.

The process of giant cell formation in the case of agar injections, as shown by these experiments, may be summarized as follows: coincident with and following an exudation of polynuclear leucocytes and serum there is a migration of endothelial cells from the blood stream through the vessel walls in the neighborhood of the agar. There is an extensive proliferation and hypertrophy of all endothelial cells fixed or free in the neighborhood of the injection. The free endothelial cells, and possibly some of those lining the blood vessels becoming free, migrate to the surface of the agar and, surrounding small masses, coalesce to form giant cells. Proliferation and coalescence continue actively for at least twenty days, with the result that the injection becomes surrounded with a dense wall of endothelial and giant cells, and outside of them there is formed a capsule of young connective tissue cells.

[These experiments were begun at the suggestion of Dr. S. B. Wolbach, and most of the work was done under his immediate supervision. The work was completed under the direction of Dr. F. B. Mallory.]

EXPLANATION OF PLATES.

PLATE I.

Figs. 1, 2, and 3 show emigration of mononuclear leucocytes of endothelial origin through the walls of blood vessels. In Fig. 1 a cell is seen entering the vessel wall; in Fig. 2 a cell is seen half way through, and in Fig. 3 a cell is emerging. Rabbit 3, two days, x 1,000.

Fig. 4 shows a mitotic figure in the endothelium of a vessel wall. Rabbit 3, two days, x 1,000.

Fig. 5 shows a mitotic figure in an endothelial cell which touches the agar at one point. On one side is a group of coalescing cells, on the other, a newly formed giant cell. Rabbit 3, five days, x 1,000.

Fig. 6 shows an endothelial cell in mitosis near the agar. Rabbit 3, five days, x 500.

PLATE II.

Fig. 7 shows several typical free endothelial cells near the agar, one of them in mitosis. Rabbit 1, 14 days, x 500.

Fig. 8 shows a giant cell which has ingested a small mass of agar; next to it a group of coalescing endothelial cells; at the other end of the cut an endothelial cell in mitosis. Rabbit 1, twenty days, x 500.

Fig. 9 shows a mass of agar surrounded by endothelial cells. Rabbit 3, four days, x 300.

Fig. 10 shows groups of endothelial cells closely assembled around bits of agar. Rabbit 3, five days, x 500.

Fig. 11 shows a ring of endothelial cells surrounding a small mass of agar. Rabbit 1, eighteen days, x 500.

Fig. 12 shows a row of newly formed giant cells in contact with a mass of agar; near them are many free cells. Rabbit 3, six days, x 300.

NOTES ON THE LATER STAGES OF THE REPAIR OF KIDNEY TISSUE (DOG) WITH SPECIAL REFERENCE TO PROLIFERATION OF THE PELVIC EPITHELIUM AND HETEROPLASTIC BONE FORMATION.*

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An account has been given in an earlier communication,[1] in collaboration with Dr. J. A. Sampson, of the general effect of the reduction of kidney substance and of the coarser changes in the remaining kidney tissue. In the present communication some of the more prominent histological changes are described briefly, with special reference to certain peculiar proliferative changes in the pelvic epithelium and the occurrence of heteroplastic bone formation.

The repair of kidney tissue after various forms of injury has been extensively studied. Marchand[2] gives a very complete summary of the literature to 1901 and to this summary those interested in the earlier literature are referred. The most complete investigation is that of Barth,[3] who studied the kidneys of the guinea-pig, rabbit, and dog after the removal of wedges of tissue or the excision of one or both poles. He found, after two days, mitotic figures in the intact epithelial cells adjacent to the necrotic epithelium; by the fourth day, the new connective tissue formation in the neighborhood of the wound was well marked. This new tissue forced its way between the tubules into the necrotic tissue and also penetrated from the capsule. The adjacent tubules were filled with homogeneous masses and their cells showed numerous mitotic figures which were found also in the connective tissue and endothelial cells. The changes preliminary to scar formation were completed by the eighth to the eleventh day.

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*Aided by a grant from the Rockefeller Institute for Medical Research. This investigation was begun in the Bender Laboratory, Albany, N.Y., and completed in the Carnegie Laboratory of the University and Bellevue Hospital Medical College, New York City. Received for publication Dec. 6, 1908.

As the scar development progressed, narrow newly formed tubules directly continuous with the old tubules, both straight and convoluted, penetrated the new tissue. These, however, were considered functionless. Sometimes in sclerosis of large areas, tubules were found, the cells of which were increased in size. In the same areas enlarged glomeruli were also present. Newly formed glomeruli could not be demonstrated. This was true also of the compensatory hypertrophy in the uninjured tissue. The pelvic epithelium near the incision was greatly thickened and presented bulging processes which impinged upon the scar. In one kidney, in which a pelvic fistula occurred, the pelvic epithelium lined the wall of the fistula.

These observations indicate that a regeneration of renal parenchyma may occur in the scar as well as a restitution of lost parenchyma by a compensatory hypertrophy. There is, however, according to Barth, no formation of new glomeruli as was claimed in the earlier work of Pisenti, Tuffier, Paoli, and Kümmell. Upon this point Barth has the support of practically all later investigators with the exception of Galeotti and Villa-Santa,[4] who describe the formation of new glomeruli and convoluted tubules in young animals in which the kidney is not completely developed. On the other hand, in fully developed animals they observed no numerical increase but merely an increase in size. An increase of straight tubules was not found in either young or old animals.

Peipers admits the new formation of straight tubules of both medulla and cortex, but denies a similar growth of convoluted tubules. According to most investigators the straight tubules penetrate the scar more frequently than do the convoluted tubules and some describe the former process only (Mattei). This regeneration, moreover, is seldom complete, as the new cell masses usually undergo degeneration.

The material upon which the present study is based consists of portions of nineteen kidneys. The operative procedure responsible for the injury consisted of the removal of the lower pole, approximately one-half of the kidney, at one operation, later a similar reduction of the opposite kidney, and in some instances after varying periods of time the further diminution of the remaining substance. In all instances the incision passed through the pelvis. The bleeding was controlled by mattress sutures of catgut and silk passing through the entire kidney substance from capsule to capsule and through the upper portion of the pelvis. Owing to multiple operations in some instances, the material for study includes portions of nineteen kidneys from fourteen dogs. These may be divided into three groups. One group of three represents early repair at six, seven, and ten days; a second group of fourteen, repair from the twenty-second to the fifty-seventh day (22, 30 [2], 33, 35 [2], 37, 41, 48 [3], 55 [2], and 57 days) and a third group of two, repair at the end of five and a half months (159 and 164 days).

As this material contains no repair lesion of earlier date than the sixth day, the initial changes of necrosis, exudation, and the early formation of granulation tissue cannot be considered. For the same reason the early stage of the regeneration of renal parenchyma and the details of mitosis, which have been fully described by Podwyssozki[5] and by Thorel,[6] have been omitted. Attention has been given, therefore, mainly to the changes which occur in the tubules and glomeruli in that part of the kidney in relation with the scar tissue, the object being to determine the ultimate results of repair rather than the initial changes. The question of vicarious hypertrophy will be considered only briefly, for although theoretically such a hypertrophy should have occurred, careful comparative measurements of the portion of the kidney removed and of that remaining fail to show a hypertrophy except in one animal.

The earlier preparations of the sixth, seventh, and tenth days show definite areas of anemic necrosis freely infiltrated with leucocytes and with moderate deposition of lime salts. Between the normal tissue and the zone of necrotic and calcified tubules a very abundant granulation tissue has penetrated. The cells of the tubules in the neighborhood of the necrosis are small and granular and the lumina of these tubules are filled with desquamated epithelial cells and hyaline casts. Back of this zone the straight tubules show evidence of proliferation of their lining epithelium. Many tubules are seen in which the lumen is occluded by cells having normally staining nuclei and well preserved protoplasm. In these cells mitotic figures cannot be demonstrated, but the arrangement and general appearance can be explained only by active proliferation.

At the twenty-second day the connective tissue formation is well advanced. The straight tubules at the border of this connective tissue, which has replaced the necrotic tissue, appear as solid masses of cells in which cell outlines are not distinct. These cells have nuclei rich in chromatin and although no mitotic figures can be seen they are certainly newly formed and not the result of desquamation. The smaller isolated masses suggest giant cells and are probably similar to the formations described by Podwyssozki. They also resemble the multinucleated masses which I have pictured elsewhere in a study of the repair of liver tissue.[7] Hyperplasia of the pelvic epithelium is present and also a small area of bone formation. In areas where small masses of kidney parenchyma are shut off from the remainder of the kidney by scar tissue, the glomeruli show greater tendency to persist than do the tubules; indeed, frequently, only glomeruli persist. This is probably due to a better blood supply and analogous to the persistence of the islands of Langerhans in chronic interstitial and congenital syphilitic pancreatitis.[8] Such glomeruli always present a thickening of Bowman's capsule and usually show evidence of atrophy.

The kidneys of the thirtieth, thirty-third, thirty-fifth, and thirty-seventh days may be considered together. Solid cell processes definitely continuous with the straight tubules are seen in the scar but no evidence of proliferation of the convoluted tubules. Some of the solid cell processes show bulbous swollen ends (see Fig. 1); others show a crescent-like proliferation on one side and in places the nests of cells formed by proliferating tubules give the alveolar appearance of carcinoma. Not infrequently these cell processes contain cyst-like spaces and offer evidence of degeneration. The pelvic epithelium is thickened and has a papillary arrangement, and in places apparently invades the subepithelial tissues. Atrophied glomeruli are still abundant in the scar tissue. Bone formation beneath the pelvic epithelium occurred twice in this period.

The kidneys of the forty-first, forty-eighth, fifty-fifth, and fifty-seventh days present no evidence of the primary necrosis except in the occurrence of calcified tubules. The scar tissue is compact. The solid cell processes and masses are no longer seen as definitely proliferating cell groups, but present evidence of degeneration. They show definite hyaline, mucoid and hydropic degeneration, and are frequently cystic (see Fig. 2). This is evident in the scar tissue generally, but more especially in that portion adjacent to the lower half of the pyramid. Solid cell processes without such degeneration are rare. On the other hand there is nothing to suggest that these solid processes have been transformed into functioning tubules. The glomeruli of the scar tissue are more or less atrophied or hyaline and closely grouped. They appear to be increased in number, but this appearance is probably due to the contraction of the scar. That these glomeruli are newly formed is contraindicated by the frequency of atrophy and fibrous thickening, and by the absence of tubules in connection with them.

At this stage the absorption of catgut sutures is well advanced. Connective tissue surrounds and penetrates the strands, and giant cells are abundant. There is no evidence of any relation between this process and the formation of new bone seen in one of the kidneys of this period.

The oldest lesions of this group (fifty-five and fifty-seven days) demonstrate definitely the disappearance of the newly formed cell masses. A few of these, of evident straight tubule origin, are seen at the border of the scar but none are present in the midst of the scar tissue.

It would appear from this summary that the power of proliferation decreases from pelvis towards cortex. The growth of pelvic epithelium is very abundant and excessive; of the straight tubules of the pyramid less marked but definite; of convoluted tubules, doubtful, and of the formation of new glomeruli there is no evidence whatever.

The latest stage of the scar is seen in two kidneys of the one hundred and fifty-ninth and one hundred and sixty-fourth days respectively. Along one end of each kidney is the remnant of a scar containing atrophied glomeruli but no tubules. This scar tissue is not at the pole but is pushed down into the hilum of the kidney and lies parallel with the pelvis. Very definite evidence of lengthening of the pelvis and lateral growth of the kidney is present. The study of the scar itself adds nothing new to what has been presented. The general hypertrophy of these kidneys is, however, of interest. The animal is the only one whose kidneys showed an increase in size as determined by measurements and plotting of diagrams. The animal lived one hundred and sixty-four days, three times as long as any other animal of this series, and for three months was boarded outside the laboratory under the best possible hygienic surroundings. It is possible that these two factors may be of great importance in determining renal hypertrophy, for all other animals were confined to the laboratory kennels and were killed after shorter periods, of three to eight weeks. In the kidneys of some of the latter animals the remaining kidney fragment as compared with the excised fragment shows some evidence of increased size of glomeruli and tubules, but always in small areas and not sufficient to cause a general increase in size capable of being determined by measurements. The one hundred and fifty-nine and one hundred and sixty-four day kidneys, however, showed a return to normal size and contour after the removal of one-half their substance. The question arises whether this increase is due to simple hypertrophy or to a true new formation of renal parenchyma.

Comparison of the hypertrophied kidney, with the excised portion, offers no evidence of the latter. Actual count of the glomeruli in comparable fields gives interesting results. If, as has been done by Barth, the number of glomeruli in a given field of the excised portion is compared with the number in a similar field of the hypertrophied portion, it is found that the former greatly exceeds the latter. For example, the number of glomeruli in the excised portion of the right kidney of the animal under consideration, in a single field (3, obj. 1, oc.) immediately below the capsule, varies from sixteen to twenty-two (average of ten fields, 19), while the corresponding hypertrophied fragment gives five to twelve per field (average of ten fields, 10). The figures for the left kidney are practically the same, an average of seventeen against eight. This points definitely to a spreading of the kidney tissue as the result of the hypertrophy of its epithelial elements. Although no new formation of glomeruli could be demonstrated it is noteworthy that the nuclei were more abundant in the glomeruli of the hypertrophied kidney as compared with the excised portion. There is, however, no evidence of immature glomeruli such as are seen in the actively developing kidney of the new-born.

Changes in the epithelium of the pelvis. — The changes in the epithelium of the mucosa of the pelvis in these kidneys are worthy of special mention. In all kidneys this epithelium was thicker than that of the normal excised portion. Not infrequently it took on a papillary growth, and in other instances penetrated the scar tissue surrounding the pelvis to such an extent as to resemble an invasive growth. This invasion, when it involved the pyramid, was not excessive, and was limited to two to six epithelial masses. On the other hand, the invasion of the scar tissue of the lower side of the pelvis, that is, the portion of the pelvis in relation with the ureter, was in three instances (thirtieth, thirty-fifth, and forty-eighth days) so diffuse as to resemble the histological appearance of a rapidly growing carcinoma. In these areas twelve to sixty epithelial masses could be counted. The ingrowth was usually in the neighborhood of the injury, as shown by the adjacent scar tissue, and directly continuous with the moderately hyperplastic pelvic mucosa. In all, the solid masses of epithelial cells had a definite alveolar appearance with, as a rule, little stroma. The cells were large with considerable protoplasm, a vesicular nucleus, and of the general character of squamous epithelium. The masses varied in size from those of a width of seven to eight cells to those occupying an entire field of a low power lens (3, obj. 2, oc). In one preparation (thirtieth day) the alveolar stroma was very definite and within two of the epithelial masses the central cells were compressed, had a concentric arrangement and took a faint eosin stain; a suggestion of the formation of "epithelial pearls" (see Fig. 3). The interpretation of this proliferation is difficult. It may be merely an example of overproliferation in the repair of the defect in the pelvic mucosa. On the other hand, in view of the decided resemblance to carcinoma, it is of interest in connection with those theories of tumor formation which assume injury, or irritation, or the loss of resistance in adjacent tissues as an etiological factor. Its analogy to the occurrence of cancer in scar tissue or chronic ulcers of the skin, as in the so-called Marjolin's ulcer, is evident.

Bone formation. — True bone was found in the scar tissue of the kidney in six animals of this series. In three animals in which but one kidney was reduced it was found at periods of twenty-two, thirty-five, and forty-eight days after operation; in two animals with double nephrectomy, in one kidney of each on the fifty-fifth and one hundred and sixty-fourth days respectively. In a sixth animal with multiple operations it was found in the portion of the left kidney removed on the thirty-third day and in the right, at autopsy, on the forty-eighth day.

The occurrence of heteroplastic bone formation in scar tissue has been reported not infrequently. The general literature of the subject, with special reference to the formation of bone in sclerotic blood vessels, has been presented admirably and completely in the recent papers of Bunting[9] and Buerger and Oppenheimer.[10] The most frequent seat of new bone formation appears to be the sclerotic artery. Next in order come calcified foci in the lung and it occurs not infrequently in the dura mater. Occasionally, the condition is found also in the lymph nodes, tonsils, liver, myoma of the uterus and tumors of the adrenal.[11]

A thorough search of the literature fails to reveal references to its occurrence in the kidney of man or of any animal except the rabbit. In this animal it has been found by several investigators in connection with the series of changes which follows ligation of the renal vessels, a procedure which, as first pointed out by Litten, is followed by a widespread deposition of lime salts in the necrotic kidney tissue. In such kidneys the circulation of the capsule is not disturbed and from the latter, and probably also from the tissues about the pelvis, an ingrowth of connective tissue occurs. These two factors, calcification and young granulation tissue, appear to be essential to the formation of new bone. The occurrence of heteroplastic bone in the kidney, under such circumstances, was first observed by Sacerdotti and Frattin[12] in three out of four animals three months after ligation of the pedicle. Their observation has been confirmed by Poscharisskey,[13] Maximow,[14] and Liek,[15] who injured the kidney in like manner, and by Harvey,[16] who found necrosis and bone formation in the kidney as the result of interference with its circulation by accidental pressure. Aside from these observations I can find no references to bone formation in the kidney.

In the dog's kidneys under consideration the bone formation occurred as thin lamellæ in the scar tissue immediately beneath the mucosa of the pelvis and, when of any length, exactly parallel to the mucosa (see Fig. 4). It occurred usually in the angle or in the lower portion of the pelvis near the origin of the ureter. It was never found in the scar tissue in direct relation with the kidney substance proper, despite the fact that more or less calcification was usually present in the latter location. This relation of the new bone to the pelvis has been noted also by Sacerdotti and Frattin, Maximow and Liek. For this peculiar localization there is no explanation, although Liek thinks it has some relation to the layer of coarse connective tissue with elongated nuclei, which lies immediately beneath the pelvic epithelium and is found nowhere else in the kidney. These areas of bone varied in size from masses, at the twenty-second day, barely filling the field of a high power lens (7, obj. 1, oc.) to one, at the one hundred and sixty-fourth day, which was eight times that size. As a rule but one mass was found in a section, but occasionally two were present. Although secondary marrow spaces were sometimes present in the bone placques, in none could the cellular elements of red marrow be demonstrated, which is not in accord with observations on the rabbit (Maximow, Liek). Haversian canals could be demonstrated readily.

Recent writers upon the subject of heteroplastic bone formation have practically abandoned the metastatic and displacement theories, and ascribe the formation of bone, in unusual locations, to one or the other of the following processes: [17]

1. Conversion of connective tissue into a callus or osteoid tissue with subsequent conversion into bone.

2. Resorption of calcified material by vessels and osteoclasts, the formation of a marrow space, and the deposit of bone in its periphery by an osteoblastic membrane derived by metaplasia from the connective tissue accompanying the vessels.

The new bone formation here described is apparently the result of the first of these processes. Lime placques could not be demonstrated in or about the areas of new bone, and in fact calcification, although frequently present in the necrotic tissue of the kidney proper, was never found in the scar tissue about the pelvis. Nor could young granulation tissue be found penetrating these areas or in close relation to them. The bone ground substance was laid down either in the practically normal coarse connective tissue beneath the pelvic mucosa or in the coarse scar tissue in close relation to it.

In other words, the process was analogous to the normal deposition of bone in the bones of the head and face, in which process osteoblasts are the result of a transformation of the connective tissue cells.

Liek states that the bone which develops after ligation of the kidney is not permanent, but undergoes necrosis and that this necrosis is complete at about ten months. Maximow notes also the disappearance of the new bone at the end of a year. Liek places the beginning of necrosis at about the hundredth day. This is possibly so, but in one kidney of this series well preserved bone showing no evidence of necrosis was present one hundred and sixty-four days after the original injury to the kidney.

Liek failed to find bone in the ligated kidney of the cat and, in his discussion of the non-occurrence of bone formation in the kidneys of animals other than the rabbit, offers as a possible explanation of its frequence in the latter animal the high calcium content (1.62) of its blood (v. Kossa).[18] That this explanation does not hold for the dog, the calcium content of whose blood is only .077, is evident from the observations here made.

SUMMARY.

1. The repair of kidney tissue at the site of an injury due to partial extirpation, as seen at the sixth to tenth days, includes the late phenomena of exudation, proliferation of the tubular epithelium, and the development of granulation tissue leading to a definite scar.

2. In and about this scar tissue (twenty-second to thirty-seventh days) is seen definite evidence of proliferation of the straight tubules, which appear for the most part as solid cell processes, often with bulbous ends. These disappear later (fifty-five, fifty-seven, and one hundred and sixty-four days) or are present as cystic enlargements, and apparently never become functioning structures. Undoubted new formation of convoluted tubules or of glomeruli has not been observed. The glomeruli, however, persist in the midst of the scar tissue

when all tubular structures have been destroyed but present evidence of atrophy and hyaline degeneration.

3. In the single instance in which compensatory hypertrophy of the kidney had occurred (one hundred and sixty-four days) and the organ had resumed, approximately, its normal contour, the restitution was due apparently to simple hyperplasia of the tubules and not to new formation of tubules or glomeruli.

4. Proliferation of the epithelium of the pelvic mucosa is the rule, and in some instances this epithelium penetrates the subjacent connective tissue and gives rise to large epithelial areas simulating an invasive growth.

5. Heteroplastic bone formation limited to the connective tissue immediately beneath the pelvic mucosa is a not uncommon condition.

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EXPLANATION OF PLATE III.

Fig. 1. — Solid cell processes, of straight tubule origin, occurring in the scar tissue; the central mass shows a characteristic bulbous termination.

Fig. 2. — (A) Hyaline degeneration and (B) cystic transformation of solid cell masses.

Fig. 3. — Invasive growth of pelvic epithelium. (A) cavity of pelvis; (B) epithelial lining of pelvis; (C) invasion of scar tissue.

Fig. 4. — (B) Bone formation in scar tissue beneath (A) pelvic epithelium; secondary marrow spaces abundant.

ON THE INCREASED INFECTIVE POWER PRODUCED IN BACTERIA BY SENSITIZATION WITH NORMAL SERUM OF THE SAME SPECIES.*

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In the study of immunity and the interrelation between bacteria and the animal organism, many papers have been contributed to the literature of the subject which form links in the chain which must sooner or later be joined to encompass the whole subject. In that field of this work, which has to do with the interaction of serum on microörganisms, there are isolated studies which are open to the objection which may be passed on so much of our experimental work; viz.: that the study has been carried on with pathological or extraordinary material without the investigator first determining what happens when the same work is done with normal material.

E. R. Baldwin[1] reported at the meeting of the Association of American Physicians in May, 1907, that in rabbits receiving intracorneal inoculations of tubercle bacilli opsonified with immune cow sera, the eyes inflamed sooner and the disease spread into the cornea farther than in his control animal. This was contrary to theory, in accordance with which, upon the basis of Wright's discovery of the so-called opsonins in the blood, and the generally accepted theory of their action, the bacteria being opsonified before entrance into the body should have produced a less severe infection than controls.

Cole,[2] without referring to Baldwin's paper, later announced a similar phenomenon with foreign normal serum. He noticed that mice inoculated with a mixture of pneumococci and normal rabbit or pigeon serum died before controls receiving the same dose of pneumococci with physiological salt solution.

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* Received for publication Dec. 16, 1908.

Opie,[3] in exhaustive studies on the relation of white blood cells of different types to bacterial infection, has shown that leucocytes obtained by injecting aleuronat into the pleural cavity and injected into animals with tuberculous pleurisy had the power of inhibiting the progress of the disease. He used dogs for his experiments and worked mainly in the pleura.

Bail,[4] and his followers and co-workers, following the suggestion of Ainley Walker[5] and Welch,[6] in his Huxley lectures, have for the past few years been presenting a mass of work to prove that microörganisms in the animal body develop an aggressive power which enables them to make a more strenuous fight against subsequent hosts in which they may become lodged. They worked with cholera, typhoid, dysentery and pneumonia organisms. They found that animals inoculated with one of the above organisms together with a bacteria-free peritoneal exudate obtained from a previous inoculation died sooner than animals receiving the dose of bacteria alone.

Bezzola,[7] studying the same phenomenon with typhoid bacteria found that typhoid bacilli allowed to remain in contact with bacteria free peritoneal exudate (from a previous typhoid inoculation, inactivated by heating to 60° C.) for one hour at 2° C., and inoculated with immune typhoid serum into other animals died, while controls receiving a like dose but in contact with physiological salt solution usually lived.

Carapelle and Gueli,[8] working with B. typhosus, Staphylococcus pyogenes aureus, Bacterium coli and Bacillus prodigiosus, attempted by cultivation in vitro in normal active serum to show that pathogenic organisms increased their virulence and that saprophytic or non-pathogenic organisms developed a pathogenicity. It would be impossible to describe the work in detail here, but they were able to demonstrate the above statement. It should be remembered that the organisms were always incubated in fresh normal serum previous to inoculation.

During the past year we have carried on, in the Laboratory of the Tuberculosis League, a series of experiments which have a very definite bearing upon all of the above work, and which may not only explain the phenomena mentioned but may later help to correlate some of the immunity work at present in progress. Our work has been a study of the interaction of microörganisms, normal serum and leucocytes and its effect on infection. Animals used as controls received the same dose of the microörganisms and the volume of the dose was made up by adding two equal volumes of sterile .85 per cent physiological salt solution. The serum and leucocytes were always fresh and from the same species and individual, except where otherwise stated. The serum was obtained in Wright capsules and separated by allowing the blood to clot and centrifugalizing. The leucocytes were obtained according to the method of Wright by collecting the blood in a solution of 1.25 per cent sodium citrate in .85 per cent physiological salt solution, and, after centrifugalizing, pipetting off the supernatant fluid and washing the residue in .85 per cent physiological salt solution. The so-called leucocytic cream was obtained after removal of the supernatant fluid.

The first series of rabbits received intracorneal inoculations of tubercle bacilli of the human type H 37, a strain kindly given us by E. R. Baldwin of Saranac Lake.

Experiment A:

Buck Rabbit I. — Weight, 1,910 grams. Tubercle emulsion + serum + leucocytes. Mixture incubated at 37° C. for thirty minutes and inoculated.

Buck Rabbit II. — Weight, 1,980 grams. Tubercle emulsion + serum + leucocytes. Mixture immediately inoculated.

Buck Rabbit III. — Weight, 2,230 grams. Tubercle emulsion + .85 per cent nacl + .85 per cent nacl. Mixture immediately inoculated.

None of the eyes showed any acute reaction, such as conjunctivitis, after the inoculation.

The first sign of reaction was a beginning hyperemia in the sclera above the point of inoculation in the cornea, which was more marked in Rabbit No. 3. This was followed by an intracorneal penetration of the blood vessels which was first obtained in No. 3. With the exception of the earlier reactions in No. 3, the lesions remained about the same and little difference could be noted.

Rabbit No. 1 was autopsied four months afterwards, but no extension of the disease could be traced. Rabbits Nos. 2 and 3 are still alive.

Our next series of experiments was to study a localized infection by inoculation of the liver. These inoculations were unsuccessful in reaching the liver, the animals receiving intraperitoneal inoculations. These experiments were carried on in rabbits all of the same litter and mostly of the same size. Each rabbit received one-twelfth milligram of tubercle bacilli H 37 with the various combinations of serum, leucocytes and physiological salt solution mentioned below. Individual leucocytes and serum were used in every case. Dose was .3 cubic centimeter, equal volumes of each constituent being used.

Experiment B:

Rabbit IV. — Tubercle emulsion + serum + .85 per cent nacl. Mixture immediately inoculated between ninth and tenth ribs of the right side into abdominal cavity.

Rabbit V. — Tubercle emulsion + .85 per cent nacl + .85 per cent nacl. Mixture immediately inoculated between ninth and tenth ribs into abdominal cavity.

Rabbit VI. — Tubercle emulsion + serum + leucocytes. Mixture incubated for thirty minutes at 37° C. and inoculated.

Rabbit VII. — Tubercle emulsion + serum + leucocytes. Mixture immediately inoculated.

Rabbit VIII. — Tubercle emulsion + serum + .85 per cent nacl. Mixture incubated for thirty minutes at 37° C. and inoculated.

None of the animals showed any evil effects after the inoculation. All were autopsied thirty days later with the following results:

Experiment B:

Rabbit IV. — Tubercle emulsion + serum + .85 per cent nacl, immediately inoculated.

Small tubercle just beneath the skin two millimeters in diameter; larger tubercle between ninth and tenth ribs about eight millimeters in diameter. On opening abdominal cavity, inoculation did not reach the liver; scattered miliary tubercles through greater omentum; no enlargement of mesenteric glands. Scattered miliary tubercles on central portion of the diaphragm; on opening thoracic cavity, pericardium shows few miliary tubercles; the lungs are free and no enlargement of bronchial glands. Above examination confirmed microscopically.

Rabbit V. — Tubercle emulsion + .85 per cent nacl + .85 per cent nacl.

Small tubercle beneath skin about two millimeters in diameter; larger tubercle between ribs five millimeters in diameter; small tubercle at lower border of right lobe of liver. Microscopic examination shows evidence of extension of tubercles into adjoining liver tissue.

Rabbit VI. — Tubercle emulsion + serum + leucocytes. Mixture immediately inoculated.

Tubercle on skin two millimeters in diameter; larger tubercle on thoracic wall four millimeters in diameter; inoculation missed the liver; odd scattered miliary tubercles in greater omentum. Thoracic cavity and contents free from tuberculosis. Microscopic examination confirms the above.

Rabbit VII. — Tubercle emulsion + serum + leucocytes. Mixture incubated for thirty minutes at 37° C.

No tubercle beneath skin; tubercle between sixth and seventh ribs three millimeters in diameter; inoculation did not reach the liver; five or six cyst-like bodies in greater omentum; scattered miliary tubercles over abdominal side of central portion of diaphragm. Thoracic cavity and contents free from tuberculosis.

Rabbit VIII. — Tubercle emulsion + serum + .85 per cent nacl. Mixture incubated for thirty minutes at 37° C.

Tubercle beneath the skin; tubercle between sixth and seventh ribs about eight millimeters in diameter; on opening abdominal cavity, numerous miliary tubercles in greater omentum as well as through mesentery; over peritoneal covering of lower portion of small intestine; all over large intestine except pelvic portion, being more marked over large than small intestine; large tubercle eight millimeters in diameter at pyloric end of stomach on greater curvature evidently point of inoculation; miliary tubercles on spleen; scattered tubercles throughout liver; miliary tubercle on hepatic diaphragmatic fold and abdominal surface of diaphragm; on opening thoracic cavity, lungs are studded with scattered miliary tubercle. Microscopic examination confirms the above.

These post-mortem examinations were quite complete and to save space negative results are not mentioned in detail.

To classify the above according to degree of infection they would probably come in the following order: Rabbit VIII., IV., VI., V., and VII. The difference between V., VI., and VII. are slight, the inoculation being confined to the liver in V., and not intraperitoneally, probably explains the absence of tubercles in the omentum. The general miliary infection in Rabbit VIII. stands out very clearly and is in marked contrast to Rabbit VI., receiving incubated leucocytes and serum instead of incubated serum alone.

Experiment C:

These experiments are a repetition of Experiment B with an added combination of bacteria, leucocytes, and serum. Inoculated five young rabbits with one-twelfth milligram of tubercle bacilli strain H 37. In these experiments the serum and leucocytes were obtained from one rabbit. The volume of the dose was .3 cubic centimeter, using equal volumes of each constituent.

Rabbit IX. — Weight, 910 grams. Tubercle emulsion + .85 per cent nacl + .85 per cent nacl. Mixture immediately inoculated.

Rabbit X. — Weight, 1,055 grams. Tubercle emulsion + serum + .85 per cent nacl. Mixture immediately inoculated.

Rabbit XI. — Weight, 1,130 grams. Tubercle emulsion + serum + .85 per cent nacl. Mixture incubated for thirty minutes at 37° C.

Rabbit XII. — Weight, 750 grams. Tubercle emulsion + serum + leucocytes. Mixture incubated for thirty minutes at 37° C.

Rabbit XIII. — Weight, 1,305 grams. Tubercle emulsion + serum. Mixture incubated for thirty minutes at 37° C., then leucocytes added and immediately inoculated.

Animals showed no evil effects after inoculation and were chloroformed and autopsied thirty days afterwards. The following results wereobtained:

Rabbit IX. — Tubercle emulsion + .85 per cent nacl + .85 per cent nacl. Mixture immediately inoculated.

Tubercle on skin two millimeters in diameter; tubercle on thoracic wall four millimeters in diameter. On opening abdominal cavity, scattered miliary tubercles over anterior parietal peritoneum, very much thicker over parietal peritoneum in iliac fossæ; miliary tubercles throughout greater omentum and mesentery, over large bowel; few tubercles on spleen; few tubercles in hepatic diaphragmatic fold and over abdominal surface of diaphragm; largely confined to central portion. Thoracic cavity and contents free from tuberculosis.

Rabbit X. — Tubercle emulsion + serum + .85 per cent nacl. Mixture immediately inoculated.

Skin tubercle four millimeters in diameter; thoracic wall tubercle four millimeters in diameter; absence of tubercles on parietal peritoneum; scattered tubercles through greater omentum and mesentery very thick in greater omentum; one small tubercle in liver two millimeters in diameter. Scattered miliary tubercles over abdominal surface of diaphragm. Large tubercles at pyloric end of stomach on greater curvature measuring 2 cm. x 1 cm. x 1.5 cm. Outer surface smooth with exception of two small spots; no tubercles on spleen; thoracic cavity and contents free from tuberculosis. Large tubercle on stomach, was probably point of inoculation and aided in confining the infection.

Rabbit XI. — Tubercle emulsion + serum + .85 per cent nacl. Mixture incubated for thirty minutes at 37° C.

Small tubercle on skin two millimeters in diameter; large tubercle on thoracic wall five millimeters in diameter. On opening abdominal cavity, anterior parietal peritoneum is rather thickly studded with miliary tubercles which are very thick in iliac fossæ; scattered miliary tubercles over peritoneum covering bladder and kidneys; ascending and transverse colon very thickly studded with tubercles; also greater omentum and mesentery. Hepatic diaphragmatic fold and abdominal surface of diaphragm both central and lateral portions show many tubercles; central portion of diaphragm has two large definite tubercles about two millimeters in diameter; tubercles over spleen; on opening thoracic cavity, lungs show few scattered miliary tubercles. The tubercles all over in this animal are larger than the other animals and show a confluence of more minute tubercles.

Rabbit XII. — Tubercle emulsion + serum + leucocytes. Mixture incubated for thirty minutes at 37° C.

Small tubercle on skin two millimeters in diameter; no tubercle on thoracic wall. On opening abdominal cavity, parietal peritoneum is free from tubercles throughout; scattered miliary tubercles over ascending and transverse colon, through greater omentum, with fewer in mesentery; few tubercles on spleen; one small tubercle on liver; two miliary tubercles in hepatic diaphragmatic fold; scattered miliary tubercles over abdominal surface of diaphragm almost entirely confined to central portion. Thoracic cavity and contents free from tuberculosis.

Rabbit XIII. — Tubercle emulsion + serum. Mixed and incubated for thirty minutes at 37° C., then leucocytes added and immediately inoculated.

Tubercle on skin four millimeters in diameter; thoracic wall free; on opening abdominal cavity, anterior parietal peritoneum rather thickly studded with tubercles which are very thick in iliac fossæ; ascending and transverse colon, greater omentum and mesentery thickly studded with tubercles; spleen covered with tubercles; liver shows few scattered tubercles; right lobe one tubercle three millimeters in diameter; middle lobe one tubercle two millimeters in diameter; many miliary tubercles in hepatic diaphragmatic fold; abdominal surface of diaphragm thickly studded with tubercles; examination of lungs showed a few scattered miliary tubercles.

It will be noted in this series of experiments that the two heaviest animals received the dose of bacteria and serum incubated for thirty minutes, while the animal weighing the least received the inoculation of emulsion, serum, and leucocytes inoculated together, and the next lightest animal was used as control. This places the differences in degree of infection at the greatest disadvantage.

To make our comparison more clear and definite, we removed portions of tissue from each animal from various organs where the infection appeared greatest and compared them. The tissues were pinned to cardboard and fixed according to the method of Watters.[9] By using an ordinary eye lens we counted the number of tubercles on the various tissues in each field of the lens and formed the following table for comparison. This does not give us an exact comparison even in number, for in Rabbit XI., as the specimen shows, the tubercles were much larger, being equal in size to three in Rabbit XI. and probably two in Rabbit IX., yet it shows a marked clear cut difference.

TABLE I.

Portion of Tissue.	Rabbit IX.	Rabbit XI.	Rabbit XII.	Rabbit XIII.
Parietal peritoneum:				
Left iliac fossæ	51	73	1	55
Anterior	6	16	0	6
Greater omentum	44	58	36	70
Mesentery	2	15	2	9
Colon	34	61	60	20
Diaphragm:				
Central	39	65	20	30
Lateral	5	51	3	4
Spleen	4	4	3	4
Total	185	343	125	198

It will be seen from the above experiment that, first: There is a striking difference between the rabbits receiving tubercle bacilli and physiological salt solution, and the rabbits receiving tubercle bacilli sensitized by their own serum for thirty minutes in the incubator. In approximate figures the resulting infection was twice as great in number of tubercles when the bacilli were sensitized than when the bacilli and physiological salt solution were injected. This difference in intensity was not only, however, one of quantity, but of quality also, for in the animals in which sensitized bacteria were used the individual tubercles were, as mentioned before, larger and often coalesced to form comparatively large masses. We would call attention here again to the fact that this great difference in degree and extent of infection must be explained by the incubation of the bacteria with an equal volume, *i.e.*, one-tenth cubic centimeter of normal rabbit serum.

Second: That in those rabbits receiving the injection of tubercle bacilli incubated with individual serum and leucocytes, the resulting infection was not only strikingly less than that in the rabbits receiving the incubated bacteria and serum without the leucocytes, being in the ratio of one to three, but was even less than the controls receiving tubercle bacilli and physiological salt solution, being in the ratio here of five to seven. That is, then, that the simultaneous incubation of bacteria, serum, and leucocytes more than counteracted the increased infective power produced by the incubation of bacteria and serum over controls receiving tubercle bacilli with physiological salt solution.

Third: That in the rabbits receiving sensitized bacteria (sensitized by incubation with fresh normal serum of the same species for thirty minutes), with the addition of leucocytes immediately before inocúlation, the resulting infection was greater than in the controls receiving tubercle bacilli and physiological salt solution alone, and much greater than that receiving the dose of tubercle emulsion incubated with serum and leucocytes together.

Fourth: That in the rabbits receiving bacteria and serum without previous incubation, which compares with Cole's experiments, the resulting infection, as he found in his experiments with pneumococci, was greater than the controls receiving tubercle bacilli and physiological salt solution, but not nearly so severe as that resulting from the injection of bacteria incubated with serum for thirty minutes before inoculation.

All these experiments, both original and controls, were quantitative, and therefore are exactly comparable with each other. There can be no doubt, therefore, that the increase in the severity of infection in the rabbits which received the bacteria incubated at body temperature outside the body, over the infection of all the other rabbits, was due to the action of the .1 cubic centimeter of fresh normal rabbit serum on the bacteria. The same increase, not nearly so marked, however, in severity of infection, was found in those rabbits in which there was simultaneous injection of serum and bacteria into the peritoneal cavity. The only difference in the preparation of these two injections was the period which was allowed for the sensitization of the bacteria by the serum outside the body. This we feel explains the difference in the resulting severity of infection.

Probably the essential requirement in obtaining the maximum intensity of infection is the exposure of bacteria to the sensitizing action of the serum at 37° C. in vitro for a sufficient length of time. Our best result was obtained by exposing the bacteria to this action of the serum for thirty minutes in the incubator. How soon the complete sensitization of the bacteria takes place, we cannot at present say, but at least there is a much greater increase in the infective power after exposure, in vitro, at 37° C. for thirty minutes, than when the bacteria are mixed with the normal serum and at once injected into the peritoneal cavity.

To review the work of previous observers, Baldwin's experiments showed that immune serum incubated with tubercle bacilli gave them an increased infective power over controls. For tubercle bacilli, at least, this property of producing increased infectivity is possessed by both normal and immune serum.

Carapelle and Gueli showed that such organisms as B. typhosus, Staphylococcus pyogenes aureus, Bacterium coli and Bacillus prodigiosus after several passages adapted themselves to growth in normal blood serum. All their organisms were grown or incubated in normal serum previous to inoculation. This makes their experiments comparable to ours and, therefore, this increased infectivity was probably due to the sensitization of the microörganism by contact with normal serum prior to inoculation.

The details of Bail's experiments are not accessible to us at this time, but we feel that our phenomenon is the factor in explanation of his results rather than the development of any new special aggressive substance.

Bezzola's experiments are rather more complicated than those of Bail. He used no control in his experiments receiving the inoculation of bacteria and physiological salt solution alone. He compared an animal which received typhoid bacilli left in contact with physiological salt solution for a definite time and immune serum of definite quantity added just prior to inoculation with an animal receiving a like dose of immune serum with bacteria which had been in contact with typhoid exudate prepared as in Bail's experiment. With the same dose of immune serum, the latter showed a more severe infection. As these experiments are not comparable exactly to ours, we do not feel justified in expressing any criticism of them at the present time.

CONCLUSIONS.

1. That the infective power of tubercle bacilli for rabbits is greatly increased by sensitization with normal rabbit serum.
2. That this sensitization may be accomplished by very minute quantities of serum (.1 cubic centimeter), and is very greatly increased by incubation of the serum and bacteria at 37° C. in vitro previous to inoculation.
3. That the increased infective power may be counteracted by exposure to leucocytes of the same species.

Complete counteraction is obtained when bacteria, serum, and leucocytes are mixed together at the same time.

4. That the power of sensitizing bacteria and thereby increasing their infective power is probably possessed alike by homologous and heterologous normal and immune serum.

5. That probably other bacteria are also capable of sensitization by exposure to serum of different origins, and that thereby their infective power is increased.

6. That the essential feature of this phenomenon is the sensitization of the bacteria by serum.

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ON THE VALUE OF THE USE OF AMMONIUM OXALATE IN BLOOD CULTURE TECHNIC.*

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The value of blood cultures as a diagnostic method has become more and more evident in recent years. With the progress of investigations along this line, it has been found necessary to use different methods for different types of cases, and in the course of the work a large variety of media has been introduced. In the ordinary blood culture it is often advisable to use four or more media.[1] In hospitals it is easier to use a variety of media, but in private practice it is not so simple. Epstein[2] suggested the use of ammonium oxalate in blood culture work for the purpose of preventing coagulation of the blood, so that the use of elaborate media at the bedside could be dispensed with and a number of methods could be employed with the blood culture after it had reached the laboratory. He pointed out in his paper that apart from this advantage it allowed of a more careful distribution of the blood in the various media. It has been the experience of all investigators who have made blood culture studies that it is difficult to distribute rapidly and properly, in various media, blood that is on the point of coagulation. He also pointed out that the coagulation of the blood adds to the bactericidal action of the serum. Chantemesse, in his work on typhoid blood cultures, reported the use of sodium citrate to prevent cogulation of the blood. Two months before the issue of the paper by Chantemesse, the ammonium oxalate method was introduced into our laboratory.

It is evident that there is an additional advantage in the use of ammonium oxalate in that the blood can be obtained from the patient by a person who is not thoroughly conversant with bacteriologic technic. And it is an advantage in private practice to have a method which prevents as much

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* Received for publication Dec. 26, 1908.

as possible the alarm of the patient or the relatives by the display of a large amount of apparatus.

It is apparent that the oxalated blood may be centrifugalized and spreads be made from the sediment and examined microscopically for bacteria in intense infections. The supernatant fluid[8] can be employed for the determination of the opsonic index.

In the Mount Sinai Hospital Reports, Volume 5, I gave our experiences with the use of ammonium oxalate solution in ninety cases. In the present paper I shall give the result of our studies up to March fifteenth of this year. The present series includes the cases given in my previous paper.

The following reagent, devised by Epstein, has been employed in our investigations:

Ammonium oxalate	2 grams
Sodium chloride	6 grams
Aq. destil.	1,000 cc.

The resulting solution is distributed into large test-tubes in quantities of ten (10) cubic centimeters measured accurately by means of a pipette or measuring funnel, and sterilized for twenty minutes on each of three successive days. It can be seen by referring to the formula that this is an isotonic solution, for it contains .6 per cent sodium chloride.

The level of the fluid is indicated on the tube by a blue pencil mark which serves as a control over evaporation, and shows at a glance the approximate amount of dilution of the blood after it has been added to the solution. The tubes are then stored in the ice-box until they are needed. The oxalate solution should never be used immediately after being taken from the ice-box, but should be allowed to stand at room temperature for some time or gently warmed. The cold solution might exert a detrimental effect on the organism sought, and by eliminating this factor the chances of a negative result are diminished. Our experience has shown us that a fresh solution is preferable, so that our stock is renewed every third or fourth week. Briefly stated this action of the oxalate is as follows: It precipitates the calcium salts of the blood in the form of calcium oxalate, thereby preventing the coagulation that would normally occur. In other words it is a decalcifying agent, and by removing the calcium base, the fibrin ferment of the withdrawn blood is rendered inert and hence clotting does not take place.[4]

Technic of obtaining the blood and of preventing its coagulation. — When possible, a vein in front of the elbow is used; under adverse conditions it may be necessary to seek one on the back of the hand or dorsum of the foot. A constriction bandage is applied above the part selected, and the field is scrubbed with soap and water. It is further disinfected with ether, alcohol, and mercuric chloride (1–500) in the order named. A sterile syringe (holding ten to twenty-five cubic centimeters) and needle are used for aspirating the blood. This having been obtained, the constricting bandage is cut by an assistant or nurse and a small dry dressing applied over the puncture wound. In our investigations part of the blood (usually four to eight cubic centimeters) was immediately introduced into a tube of the oxalate solution after having previously sterilized the mouth of the tube in the flame of an alcohol lamp. The remainder was distributed in the usual series of media. The ten cubic centimeters of ammonium oxalate solution are more than sufficient to decalcify ten cubic centimeters of blood, which was the maximum amount used in any single tube because of the danger of contamination in case the blood oxalate solution came in contact with the cotton plug. In cases where an unusual amount of blood was withdrawn, that not employed in the control set of media or in the first tube of oxalate solution was introduced into a second. The mouth of the tube containing the blood oxalate solution is then sterilized in the alcohol flame, the cotton plug replaced, and the tube gently shaken. This is sufficient to thoroughly mix the blood with the oxalate solution in cases where a moderate amount (two to five cubic centimeters) of blood has been obtained. When six to ten cubic centimeters are introduced into a single tube of the oxalate solution it has been my technic to pour the mixture into a sterile tube and back again into the original tube with the usual precautions. In this way one can be sure of a more rapid and even distribution of the blood in the oxalate solution so that all possibility of clotting is obviated, even though the coagulation time of the patient's blood be markedly diminished. On standing, the blood oxalate solution usually separates into a stratum of blood cells with a supernatant layer of turbid fluid. When later platings are made, it is desirable to pour the mixture into a sterile tube and back again into the original tube before adding the oxalated blood to the medium selected. This distributes the cells evenly in the solution, and plates of uniform color are obtained.

Media used. — In our investigations the routine blood culture set employed in this laboratory was used as a control. In most instances the same media were used in the two methods, but occasionally when there was a large supply of oxalated blood, in addition to media analogous to those of the control (*i.e.*, the routine set), special media were employed.

Our routine blood culture method, introduced by Dr. Libman,[5] consists in the use of flasks of plain bouillon (one per cent acid) and two per cent glucose bouillon (one per cent acid), together with plates poured from the blood mixed with plain agar (one per cent acid), serum agar, two per cent glucose agar, two per cent glucose serum agar, five per cent glycerine agar, and neutral plain agar. Corresponding flasks and plates from the oxalated blood were made from time to time, some at once, others at intervals of thirty minutes, one, two, three, or five hours, etc., depending upon the amount of blood withdrawn. Occasionally we have extended the intervals, plates at times being poured twenty-four to thirty-six hours after the blood had been obtained. In the intervals between platings the oxalated blood was kept in the thermostat at 37° C.

Results. — Three hundred and twenty-four blood cultures have been taken in which the method has received a fair trial. Of this number eighty-seven positive cultures are available for study. The blood cultures were made in all the usual types of disease encountered in hospital work. The following is a short list of the diseases in which the positive results were obtained: Typhoid fever, pneumonia, post-partum infections, pyelitis, pyosalpinx, infective endocarditis, otitic infections, erysipelas, local infections in various parts of the body, abscesses of various parts of the body, gall-bladder infections, osteomyelitis of various bones, glanders, and general cryptogenetic infections.

The main point of the study has been to determine the effect of the ammonium oxalate solution upon infected blood. Is this effect deleterious or not? In analyzing our results, many factors must be taken into consideration. A few of the more important ones which will be taken up are the number of organisms in infected blood in various pathological conditions; the viability of different bacteria; the bactericidal effect of the serum introduced into the oxalate solution; the physical appearances of media inoculated with oxalated blood.

The number of organisms in the infected blood. — Typhoid fever is a disease associated with relatively few organisms in the circulating blood. Twenty-six positive cultures in this series, representing one hundred and ninety cubic centimeters of blood, show a total of one hundred and seventy-two colonies, an average of less than one colony per cubic centimeter of blood. Of these twenty-six cultures the highest single count was about three colonies per cubic centimeter; the lowest, one colony from ten cubic centimeters of blood. The length of time that the disease has lasted seems to be of some consequence, more bacilli being found per cubic centimeter in the earlier stages and in relapses than late in the disease. Against the possible objection that the small number of organisms is consequent upon the bactericidal effect of the blood, is the fact that these organisms multiply in defibrinated, oxalated, laked, and clotted blood without the addition of any nutrient medium, as pointed out by Epstein.[6]

One can readily appreciate that in a given sample of blood containing very few organisms or a single bacillus, there is a large element of chance as to whether a medium of the oxalate series or of the control will be favored. In twenty-five cultures in which both methods were used, twenty-one showed growth in some media of each set. Of the remaining four cultures, two showed growth in a single flask of the control series; one was positive in the Conradi medium and a flask of bouillon; and one showed a single colony on a plate and growth in a flask. To offset these four cases the oxalate method alone was employed on four occasions; each gave a positive result. It is therefore reasonable to suppose that had all of the blood obtained in the four cases first referred to been introduced into the ammonium oxalate solution subsequent platings would have shown growth. Apparently that portion of blood used in the oxalate series in these four cases contained no bacilli, and hence the media employed remained sterile.

To emphasize still further the element of chance in transient blood infections and bacteriemias associated with few bacilli four cases in our series of streptococcemias may be mentioned. Two showed a single colony or a positive flask in the control, the oxalate series remaining negative. Of the other two, one showed three colonies on the plate of the oxalate series, the control media being negative; the other gave a positive result in a flask and plate of the oxalate series, four plates and a flask of the control being sterile. Had all of the blood obtained been introduced directly into the oxalate solution instead of only a portion, the first two cases would have undoubtedly shown growth in the platings. Most of our streptococcus infections recorded in this series have been very intense and there has been no difficulty or discrepancies encountered in comparing the two methods. A rough average of the cases of post-partum infections shows about sixty colonies per cubic centimeter of blood. Between the two extremes represented by the few bacilli of the typhoid bacteriemia and the overwhelming number of streptococci in some post-partum infections — at times five hundred to nine hundred to the cubic centimeter — all of the other blood infections can be numerically grouped. It has naturally been easier to study the value of the oxalate method in those cases where many organisms were present than when the reverse held true.

The viability of different bacteria in the solution. — This factor is not a negligible one. Most organisms isolated from the blood can be made to grow without difficulty, such, for example, as the typhoid bacillus, bacterium coli, bacillus mucosus capsulatus, micrococcus aureus, and many strains of streptococci and pneumococci. On the other hand, there are various organisms like atypical pneumococci, the attenuated streptococcus associated with endocarditic lesions, and possibly the meningococcus and gonococcus which require extreme care in isolation. The last two mentioned organisms we have not encountered in the series under consideration. In general one has some idea of the identity of the possible infecting agent from the history of the case. Therefore he can select the most favorable media before distributing the oxalated blood. If this be done there need be no doubt in most cases concerning a negative finding.

Although our experience with the attenuated organisms found in certain cases of endocarditis has been limited, we have recovered them from the blood oxalate solution in several cases. The attenuated streptococcus grows extremely poorly or not at all, even on the most favorable media. The studies in the use of ammonium oxalate in cases of subacute and chronic streptococcic endocarditis will be published later, when enough cases have been studied.

Bactericidal effect of the serum introduced into the oxalate solution. — The work of Eppenstein and Korte indicates that the typhoid bacillus develops an immunity against the bactericidal action of the blood of the host. Epstein[7] has confirmed this fact. Bacterium coli, Bacillus mucosus capsulatus, most streptococci and pneumococci, Bacillus mallei, Micrococcus aureus and albus are apparently not inhibited by any bactericidal effect of the serum, but tend to multiply in the oxalate solution. Concerning the possible bactericidal effect, we adopted the rough test of inoculating sterile oxalated blood from negative cultures with various organisms. In no instance was a later sub-inoculation from the oxalated blood sterile; in fact, we often employed this method for increasing viability and improvement in growth in dealing with attenuated organisms. Whether there was originally a bactericidal effect in the fresh blood or not is of no importance here; if it was present it may have been neutralized chemically or by dilution in the ammonium oxalate solution.

In only one instance was there any question as to the exertion of a possible bactericidal effect. This was a case of pneumonia complicating chronic endocarditis. The control media showed an average per cubic centimeter of blood of four thousand seven hundred and fifty colonies; the oxalate plates (2) which were poured twenty hours after the blood had been obtained showed only one hundred and six colonies per cubic centimeter of blood. Insufficient blood had been obtained for extended observations, and through a misunderstanding the plates from the oxalate were not poured after intervals of one and two hours as had been intended. Unfortunately the death of the patient precluded further investigation, so that criteria as to the behavior of this particular sample of infected blood over the period up to the twenty-hour plating were not possible. While this case is of interest, it does not reflect upon the method; it will be the exception, rather than the rule, when the interval in practice between the time the blood is obtained and the time it reaches the laboratory is more than two or three hours.

While conceding a possible bactericidal effect in this particular instance, although the same is quite unlikely, the patient being moribund at the time the blood was obtained, I was nevertheless able to recover ample organisms after the twenty-hour interval to permit of easy diagnosis. This was the only doubtful case; eight others in which the pneumococcus was identified show either a corresponding number of colonies in the oxalate series as compared to the control or an increase.

Appearances of media inoculated with oxalated blood. — Plates properly poured from the blood oxalate solution after admixture with any nutrient medium, present no macroscopic difference from plates poured in the usual way. Briefly summarized, the following data can be regarded as reliable: The phenomena of hemolysis as produced by the streptococcus, and often by the micrococcus aureus, are in no way interfered with by the ammonium oxalate solution. Likewise the appearance of the typhoid "type colony"[8] on the two per cent glucose agar plates of the oxalate series is identical with that on the control plates of the same medium. With the pneumococcus we have observed hemolysis on some media and zones of green coloration surrounding the colonies, on others, analogous plates in the two methods presenting identical features. With Bacillus mucosus capsulatus observations on glucose agar plates as regards the production of zones of green coloration about the deep colonies, and later zones of precipitation, indicate that corresponding phenomena are displayed in the two methods. It is therefore reasonable to assume that with other organisms we may expect to find that the oxalate method will show features in no way different from those seen in blood media poured in the usual way.

Organisms studied. — The following is the summary of bacteria isolated from eighty-seven positive blood cultures since the oxalate method was introduced:

Bacillus typhosus	29
Streptococcus	26
Pneumococcus	9
B. mucosus capsulatus	7
Streptococcus mucosus capsulatus	1

Micrococcus aureus	5
Bacterium coli	1
Bacillus mallei	2
Attenuated streptococcus	2
Bacillus proteus (atypical)	1
Micrococcus albus	1
Micrococcus zymogenes	1
Mixed infection	2

Effect of the ammonium oxalate solution on the various organisms. — In our study of typhoid infection it was not uncommon to note an increase in the number of colonies on the glucose plates poured from the oxalated blood. The data in a single case will be cited by way of illustration, for this disease being one associated with relatively few bacteria in the blood the particular medium to which one bacillus or more may be added is largely a matter of chance, as I have already pointed out.

The media included in the control series were a flask of two per cent glucose bouillon and two plates of two per cent glucose agar, each receiving about two cubic centimeters of blood. Two similar plates were poured from the oxalated blood thirty minutes after it had been obtained.

Results:

	19 hours.	48 hours.	72 hours.	96 hours.
Control series:				
Flask	Neg.	Neg.	Neg.	Neg.
Plate 1	1 "type" colony.	Same.	Same.	Same.
Plate 2	3 "type" colonies.	Same.	Same.	Same.
Oxalate series:				
Plate 1	4 "type" colonies.	Same.	Same.	Same.
Plate 2	5 "type" colonies.	Same.	Same.	Same.

We have other cases in our series showing an increase in the number of bacilli during their stay in the ammonium oxalate solution, but the case mentioned is sufficient to demonstrate this point.

Streptococcus. — It has been our experience in most instances that the streptococcus tends to multiply rapidly in the oxalate solution. Plates made after an interval of several hours generally show a progressive increase in the number of colonies:

Control Series.	Oxalate Series.
Poured at bedside:	Poured after two hours:
(1.) 86 colonies.	(1.) 50
(2.) 36 colonies.	(2.) 60
	Poured after five hours:
	(3.) 251
	(4.) 208
	Poured after nine hours:
	(5.) } Too numerous to be counted.
	(6.) }

Control Series.	Oxalate Series.
Poured at bedside:	Poured after thirty-six hours:
(1.) 1 colony.	(1.) 700
(2.) 1 colony.	(2.) 600
(3.) None.	(3.) 480
(4.) None.	(4.) 300

In five cultures in which the oxalate method was alone employed the streptococcus was isolated without difficulty in every instance.

Pneumococcus. — While this organism does not show as marked a multiplication as the streptococcus in our nine positive infections there was evidence of increase in number of pneumococci in the majority of instances.

Micrococcus aureus. — Like the streptococcus the micrococcus aureus tends to multiply in the oxalate solution. The following cases are instructive:

	No. of Colonies per Plate.	No. of Colonies per Cubic Centimeter.
Control series:		
Plate 1	87	118
Plate 2	48	
Plate 3	37	
Oxalate series:		
Plate 1 (poured at once)	....	140
Plate 2 (poured after two hours)	....	196
Plate 3 (poured after five and one-half hours)	....	212

It will be noted that the plates poured from the oxalated blood at successive periods show a gradual increase.

A case of post-partum infection, likewise due to the micrococcus aureus, shows a still more striking increase in the number of colonies:

Control Series.	Oxalate Series.
Plate 1, 264.	Poured at once: Plate 1, 294.
Plate 2, 222.	Poured at once: Plate 2, too numerous to be counted.
	Poured after two hours: Plate 3, 720.
	Poured after four hours: Plate 4, 744.
	Poured after seven hours: Plate 5, too numerous to be counted.

The oxalate method without the control has been tried with this organism and has been found to work entirely satisfactory.

Bacterium coli. — The colon bacillus shows an unmistakable tendency to multiply in the oxalate solution. There are two cases to support this point.

Bacillus mucosus capsulatus. — In two cases the oxalate method alone was employed, the bacillus being readily recovered. There was a tendency toward multiplication in the oxalate series in four cases in which a control was used, and a decided increase in number in a fifth case.

Bacillus mallei. — We were fortunate in having two blood cultures in two cases of glanders for study. In one case there was noted an increase in the bacilli while in the oxalate; in the other the oxalate series showed fewer colonies, probably due to chance.

Streptococcus mucosus capsulatus. — In investigating a case of pneumonia this organism was encountered. The blood oxalate solution was plated after an hour's interval. In the control series one flask was positive, a second flask and four plates remaining negative. There were three colonies on the four plates of the oxalate series.

Attenuated streptococcus (found in sub-acute endocarditis). — As stated above, the results of the studies in connection with this organism will be published later. It can simply be stated here that our studies thus far indicate that the ammonium oxalate method is satisfactory in such cases.

Miscellaneous infection. — The micrococcus albus, micrococcus zymogenes, the bacillus proteus, were each studied once. In every instance the organism was readily recovered from the oxalate solution.

Two post-mortem blood cultures are of interest. One was a case of suppurative cholangitis from which bacterium coli was isolated during life; the post-mortem culture showed this organism together with the streptococcus. The other culture taken after death showed three organisms on the plates — bacterium coli, micrococcus aureus, and streptococcus. From these two cases it may be deduced that the oxalate solution will be as serviceable in mixed infections as in the bacteriemias due to a single organism.

Spreads made from centrifugalized oxalated blood. — In the severe infections a clue to the case may be furnished early by such spreads. We have had some success in these examinations, especially where the streptococcus, pneumococcus, and micrococcus aureus were the infecting organisms. In the bacteriemias associated with relatively few organisms, *e.g.*, typhoid fever, the examination is necessarily useless.

CONCLUSIONS.

Our experience, as detailed above, leads us to believe that it is safe to use the ammonium oxalate method as an intermediate step in blood culture technic. We have shown that good results were obtained with all of the organisms that were encountered during the time of the study. Unfortunately the meningococcus, gonococcus, and the paratyphoid bacilli were not met with; and the streptococcus mucosus capsulatus was encountered only once. Any experiments which we may have in connection with these organisms will be published later, as will also a comparative study of the value of ammonium oxalate and sodium citrate; our present experience is decidedly in favor of the former. In the study of mixed infections no difficulty should be experienced if the distribution of the oxalated blood is made as soon as the material arrives at the laboratory. The simple method suggested by Epstein as an intermediate step in blood culture work should lead to the more general use in private practice of blood cultures as a diagnostic method.

[I wish to thank Dr. E. Libman for suggestions, and Dr. A. A. Epstein for the privilege of working out his method in detail. I am also indebted to Dr. Epstein and to Dr. J. J. Hertz for the data in some of the cases comprised in my series.]

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GENERAL SUSCEPTIBILITY IN TYPHOID AND COLON INFECTION AS SHOWN BY THE OPHTHALMIC TEST.*

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In any infection occurring in that portion of the body to which the lymphatic and circulatory systems have free access, or in which these channels are themselves invaded, general susceptibility of tissue cells to the elaborated poison frequently follows. Von Pirquet and Calmette have shown this to be true in tuberculosis by means of the cutaneous and ophthalmic tests, and, on account of the uniformity and persistency of the tissue sensitiveness of eye and skin, the diagnostic value of their work has been marked. Following the development of these tests in the diagnosis of tuberculosis, Chantemesse in 1907 demonstrated conjunctival susceptibility in typhoid fever to the toxic product of the bacillus, and showed the reaction to be specific.

The test consisted of the instillation into the conjunctival sac of a solution prepared as follows: Virulent typhoid bacilli after cultivation for eighteen hours were suspended in sterile water and sterilized at 60° C. The solution was centrifugalized, the organism collected, dried and triturated. A suspension of the triturated product was then made in sterile water and allowed to stand for three days. The disintegrated organisms were then removed and the extract precipitated with alcohol. The precipitate was dried and the powder dissolved in sterile water in the strength of two milligrams to one minim.

The use of this preparation on some seventy cases of typhoid fever gave a definite ophthalmic reaction in every case. Following the instillation of the solution into the conjunctival sac, in three hours a diffuse redness of the conjunctiva was noted, and lachrymation and exudation appeared later. The height of the reaction occurred in from six to eight hours and lasted for twenty-four hours. In some fifty

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* Received for publication Dec. 28, 1908.

cases of various diseases other than typhoid fever a slight injection of the conjunctiva occurred, which disappeared in a few hours. This was considered a negative reaction.

The use of the solution as prepared by Chantemesse in the diagnosis of typhoid fever was undertaken by the writers. Typhoid bacilli were isolated by means of cultures from the blood and the stools and grown for twenty-four hours. Precipitation of the extract of the organism with alcohol was readily obtained, but the suspension of the produced precipitate in saline solution was found to be inert in several trials. Even the sensitive conjunctiva of infants and children failed to react. On account of failure to obtain an active precipitate by means of the trituration method, the simpler technic of Hamburger was adopted.

Preparation of the solution. — A strain of virulent typhoid bacilli was grown on agar-agar for twenty-four hours in sufficient quantity. The organism was then washed from the media with physiological saline solution and the suspension incubated for four days. At the end of this period the solution became partly clear by the settling out of the organisms, which had become more or less disintegrated. The solution was then sterilized at 60° C. for one-half hour, centrifugalized for twelve hours, and the clear supernatant fluid was pipetted off into sterile tubes. The solution as advocated by Hamburger contained the toxic principle of bacilli three billion to the cubic centimeter. Some twenty-seven cases of typhoid fever were tested by him with this preparation, and positive results were obtained in every case.

We have followed this technic closely, but have found a solution used in the strength of three billion bacilli to the cubic centimeter of saline solution much too strong. A strength of one billion bacilli per cubic centimeter, or in children one of half of this strength, is better adapted for reliable results. Hamburger obtained slight reactions in a number of negative cases, and this we believe to be due to the concentration of the solution used. The number of bacilli per cubic centimeter of fluid was determined by the Wright method of standardizing vaccines. The more virulent the bacilli used, as shown by the results of infection in the clinical case and modified by as little cultivation as possible, the more powerful the extract. In using a strong solution more certain results can be obtained by diluting the extract with sterile water to one-half its strength. It retains its strength for several weeks and then gradually grows weaker until nearly inert. The less it is exposed to the air the better. If we accept the work of Vaughan, that the proteid molecule of the typhoid bacillus consists of a poisonous and non-poisonous group, and that these may be extracted by hydrolysis, then, as they are both soluble in an aqueous solution, we probably have in the extract both the toxic principle which gives the reaction, and the soluble albumin of the bacillus. How far the toxin is specific is shown by the results of Hamburger and those given below.

Character of the reaction. — With the instillation of sterile saline solution containing the bacterial albumins into the conjunctival sac, the response is rapid and characteristic. Within four hours, and often in two or three hours, the conjunctiva becomes injected, and lachrymation rapidly follows. At the end of twelve hours the reaction is at its height, and then gradually disappears, until at the end of twenty-four hours the eye appears normal, or only a little injection remains. The reaction is frequently violent, especially in the very susceptible, and at the height of the reaction the eye looks seriously affected. The inflammation rapidly subsides, however, and in our series of cases not even temporary injury has been done to the tested eye. As it has been found to be true of the ophthalmic reaction in tuberculosis, so it is true of the typhoid eye reaction, that the presence of local affections preclude the use of the test. Conjunctivitis, ulceration of the cornea and iritis are contra indications. Functional disturbance of the eye can be neglected.

That the ocular test in typhoid fever is reliable is shown by the fact that of seventy cases reported by Chantemesse a positive reaction occurred in every case, and in twenty-seven cases reported by Hamburger similar results were obtained. In our series of ninety-three clinical cases of typhoid fever, nearly all of which were proven by the Widal test or by blood culture, all but two gave a well marked reaction in about four hours after the application of the solution to the eye.

In some twenty-four control patients suffering from a variety of diseases other than typhoid fever, four gave a well marked ophthalmic reaction. These were all tubercular cases, and the reaction occurred in from two to three hours after the test was given. In three cases having clinical symptoms of typhoid fever, but not proven by the Widal test or by blood culture, the eye reactions were positive.

The failure of two proven cases of typhoid fever to give this test can only be explained by the failure of the body tissues to become sensitized to the typhoid toxin through the disintegration of the bacilli within itself. In the tubercular cases reacting to the test the possibility of a previous persistent gall bladder injection with typhoid bacilli must be borne in mind. The difference in the sensibility of different conjunctivæ is of importance in determining the presence or degree of the reaction. Meroni in testing some fifty-five cases by the method of Chantemesse obtained a positive reaction in the cases of typhoid fever, and also in some other conditions. In the total series of some two hundred reported cases of typhoid fever all but two gave a definite ophthalmic reaction to the typhoid toxin.

Period of disease at which the reaction is obtained. — The ophthalmic reaction, when it has been well marked, has occurred in our cases only during the active period of the disease. Where the destruction of the typhoid bacilli was still in progress and the body tissues were sensitized by the freeing of the endotoxin the reaction was generally well developed. In cases during convalescence after the temperature had reached normal very little, if any, reaction was obtained. Also in several cases in the first two weeks of the disease the ophthalmic test was obtained, but later it disappeared with the establishment of convalescence, and reappeared in those cases developing a relapse. From this it would seem that the union of the toxin molecule of the bacillus with the tissue cells of the body is temporary, and unless constantly renewed sensitiveness rapidly disappears. The intensity of the reaction has been greatest, as a rule, in the most toxic cases and generally in the second week of the disease. In some instances, even in adults when the solution has been used at one-half strength at the active period of the disease, the test has been very marked.

The relationship between the presence of the ophthalmic reaction, the blood culture, and the Widal test has been interesting. In a number of instances no comparison could be made as the cases tested had been in the hospital wards for some time before the eye reaction was carried out. In those instances where comparison was possible, there seemed to be little, if any, connection between the agglutination test and the ophthalmic reaction. The blood culture and the eye reaction occurred much more closely in point of time, but in either case the ocular reaction has frequently been obtained some hours or days previously to one or both of the other tests.

The eye test was always obtained at the first trial and did not develop subsequently as is the case with other tests, if the first trial was negative. Table I. shows clearly the relationship of these tests.

TABLE I.

No.	Duration of Disease (on Entrance).	Widal.	Blood Culture.	Ocular Test.	Degree* (of Reaction).	Precedence of the Ocular Test over Others.
1..	8th day.	15th day.	38th day (in relapse).	10th day.	xx	5 days.
2..	10th day.	12th day.	Negative.	14th day.	x	
3..	10th day.	36th day (relapse).	32d day.	20th day.	x	12 days.
4..	14th day.	23d day.	Negative.	17th day.	xx	6 days.
5..	7th day.	18th day.	9th day.	7th day.	xx	2 days.
6..	9th day.	13th day.	Twice negative.	10th day.	xx	3 days.
7..	7th day.	Negative.	14th day.	8th day.	xx	6 days.
8..	7th day.	9th day.	Negative.	7th day.	xx	2 days.
9..	7th day.	Negative.	Negative.	8th day.	xx	Clinically typhoid (duration 45 days).
10..	6th day.	6th day.	Negative.	7th day.	xx	
11..	9th day.	7th day.	8th day.	7th day.	x	
12..	5th day.	Negative.	8th day.	6th day.	x	2 days.
13..	35th day.	37th day.	37th day.	35th day.	x	2 days.
14..	7th day.	13th day.	0	9th day.	xx	4 days.
15..	36th day.	36th day.	0	36th day.	x	
16..	38th day.	42d day.	45th day.	42d day.	x	
17..	21st day.	24th day.	0	23d day.	x	1 day.

* xx = very marked reaction. x = moderate reaction.

From this table it is readily seen that in those instances where the ocular test was tried at the same time with the blood culture and the Widal test in the great majority of instances it preceded them in from two to ten days. In all but one instance the ocular test was substantiated by other diagnostic means. In the other cases not tabulated the test was tried days or weeks after the blood culture or the Widal reaction and no series of comparisons as to priority could be made.

It will be seen that the ocular reaction can be obtained as early as the fifth day of the disease and frequently in the first week. Reactions have also been obtained as late as the seventh week of the disease.

In six of the series of typhoid cases the Widal was not obtained, but the cases were proven to be typhoid fever by blood culture. In thirty-five instances after one or more trials the blood culture was negative, but the Widal reaction was obtained. In all these cases the ocular test was positive.

Diagnostic value of the ophthalmic reaction. — In our hands the typhoid ophthalmic reaction has proved of some diagnostic value. The test is simple, and without danger. The solution is easily prepared, and, if carefully sterilized, it can be used freely on adults or children. Its reliability is shown by the fact that ninety-six per cent of our series of typhoid cases gave a definite reaction. This test is frequently obtained several days before the Widal reaction or blood culture, and the doubtful case can frequently be diagnosed earlier than by other methods. On account of the sensitiveness of the normal conjunctiva, the solution for the ocular reaction should not be too concentrated, as error may occur in this way.

We feel that the typhoid ophthalmic test is a specific reaction due to the susceptibility of the conjunctiva to the typhoid toxin and that it may have extended use in the diagnosis of typhoid fever.

Colon ophthalmic reaction. — In carrying out the typhoid ophthalmic test on cases of typhoid fever the question of whether the conjunctival reaction was specific at once presented itself. Might not a similar reaction be produced by the toxin of the colon bacillus in typhoid cases, as well as by the infecting organism? The close relationship of these two organisms made the determination of this point desirable. The bacillus coli communis was cultivated, and, after extracting in salt solution, was sterilized and centrifugalized from the solution. A solution containing the toxic principle in the strength of one billion bacilli to the cubic centimeter of normal saline solution was obtained. The eyes of some twelve adult typhoid fever patients in the active stage of the disease were tested and a negative result obtained in all but one instance. In this case reaction was very slight and transitory. In applying the test to some six or eight cases of typhoid fever in children a slight injection lasting a few hours was obtained, but very much less marked and coming on earlier than the true typhoid reaction. It is thus seen from this limited number of tests that the reactions are not interchangeable, but that the typhoid ocular reaction is specific.

The question of the presence of an ophthalmic reaction in colon infection was then studied. For this work there were available only seven cases; one of colon cystitis, five cases of colon pyelitis and one infected arm, all but one being in adults. In six instances after instillation of the colon toxin into the eye a marked reaction occurred; in two the eye became injected, lachrymation developed and the formation of pus occurred. The reaction lasted about twelve hours, and at the end of twenty-four hours had entirely disappeared. In one case of colon pyelitis the solution was diluted one-fourth with sterile water, and no reaction occurred, probably on account of the weakness of the toxin, or because the infection was not severe enough to produce tissue sensibility. The colon ophthalmic reaction differed in no way from that obtained in the cases of typhoid fever except that it generally occurred in a shorter time and was more severe. As to whether the typhoid toxin will produce a reaction in colon infections, we can only report on one case, which is of considerable interest. A man was under treatment for pyelitis of the right kidney. The colon organism was isolated from the urine, and the colon ophthalmic reaction obtained with the use of the colon toxin. The case, however, was also suggestive of typhoid fever, for the spleen was enlarged and rose spots were present. A few days after the colon eye reaction was obtained the unused eye was tested with the typhoid toxin and a definite reaction was obtained. A number of days later the blood culture gave the typhoid bacillus;

no Widal reaction was ever obtained. A case of typhoid pneumonia gave a definite typhoid ophthalmic reaction, as well as a positive Widal. In using the colon and typhoid toxins it has not been found to be necessary to use the strain of organism isolated from that particular case in order to obtain the eye test.

TABLE II.

Diagnosis.	Reaction.	Time.	Complication.
Cystitis	x	3 hours.	o
Infected stump of forearm......	xx	2 hours.	o
Pyelitis	o	o	o
Pyelitis	xx	2 hours.	Typhoid fever.
Pyelitis	xx	3 hours.	o
Pyelitis	xx	2 hours.	o
Pyelitis	x	2 hours.	o

The value of the colon ophthalmic reaction diagnostically will probably be small, but occasionally it may give evidence as to the nature of deep seated abdominal infection.

In a series of cases in which the pneumococcus was used no results were obtained. The solutions prepared were inert in every case after several attempts. Further work, however, will be carried on along these lines. It would seem, then, that the general infections of tuberculosis, typhoid fever, and the colon bacillus give rise to a more or less marked sensibility of tissue cells. The specificity of this sensibility is of value in diagnosis and the simplicity and reliability of the methods in vogue would seem to warrant their more general use.

[We wish to take this opportunity to thank the visiting physicians of the Massachusetts General Hospital and Children's Hospital for their kindness in allowing these studies to be carried on in their wards.]

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A MUCIN—CONTAINING MULTILOCULAR CYST OF THE OVARY.*

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For many years the chemistry of the contents of ovarian cysts has been of as great interest as has been the histology and clinical course. And it might be said that as little advance toward accurate knowledge has been made in the one as in the others.

In 1852 Scherer[1] investigated the material obtained from ovarian cysts and isolated two substances that he called "metalbumin" and "par-albumin." Under the first name he reported a peculiar protein body that gave certain reactions, amongst which were the following: On carefully adding acetic acid the ovarian fluid was not precipitated but became milky and could not be cleared by filtration. Alcohol in large amount gave a stringy precipitate that dissolved on the addition of water. On the addition of hydrochloric acid alone or with potassium ferrocyanide the fluid became opalescent and cloudy. Millon's reagent gave a coagulum that became red on warming.

Paralbumin reacted quite similarly to the above. With those reagents that cause a precipitation of albumin, such as nitric acid, tannic acid, mercuric chlorid, lead acetate, potassium ferrocyanide in acetic acid or nitric acid solutions, there is formed a heavy precipitate of paralbumin. It differed from the first, however, in that acetic acid alone does not cause a precipitate and that on boiling with dilute mineral acids there is constantly obtained a strong reducing agent.

Further contributions were made by Scherer[2] and by Eichwald,[3] the latter adding but little to Scherer's descriptions.

It was then not until 1882 that the subject was again taken up. In that year O. Hammersten[4] published a long and

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* Received for publication Dec. 31, 1908.

critical article based on the results of his examination of material obtained from forty ovarian cysts.

His description of the first of the ovarian fluids examined by him is as follows: The fluid was white, very tenacious and slimy, appearing like thick mucilage. The reaction was faintly alkaline, the specific gravity 1024 and the solid contents constituted 8159 per cent. The undiluted material would not filter, but on the addition of three volumes of water it did so slowly. The filtrate was opalescent, thick, and slimy, but much less tenacious than the original substance. It reacted as follows on the addition of various reagents: Alcohol gave a long stringy precipitate that a month later was almost completely soluble in water. On boiling, the filtered fluid became strongly opalescent but did not coagulate. Even on the most careful addition of acetic acid no precipitate appeared. Tannic acid caused a precipitate that was quite different from those obtained in egg-white solutions with the same reagent. The solution was at first rather thick, then slimy, tenacious, and finally almost colloid. Acetic acid in no amount, whether large or small, caused a precipitate. When combined with potassium ferrocyanide there was no precipitate formed, the fluid becoming only thicker and slimy. Hydrochloric acid gave no precipitate, but when given with potassium ferrocyanide the fluid became opalescent and thick but did not precipitate. Nitric acid caused the fluid to become first opalescent and thick, this finally changed in color to yellow.

As a result of his thorough investigation Hammarsten concluded that the characteristic element of ovarian fluids was a substance to which he gave the name of "pseudo-mucin." He also was of the opinion that the material called metalbumin by Scherer was pseudo-mucin free from albumin, while paralbumin was a mixture of pseudo-mucin and albumin, the latter largely in the form of serum albumin. According to Hammarsten a mixture of pseudo-mucin (metalbumin) and blood serum reacts, not only to albumin reagents in general, but also to boiling as well as to alcohol, in a manner similar to paralbumin. His view is that there can be no doubt that paralbumin is a mixture of the two substances mentioned. If one considers paralbumin as such a mixture it is easy to understand the varying peculiarities of the material. It will also be readily appreciated that a sharp line cannot be drawn between paralbumin and metalbumin. As far as he could determine, the ovarian fluids contain no specific albuminous substance. There are, however, found in them very small amounts of pepton and varying amounts of globulin and serum albumin. In addition to these there is always present a specific element, one of the mucoid bodies, pseudo-mucin, and it is to this that the ovarian fluids owe their peculiarity.

Hoppe-Seyler[5] comes to a similar conclusion, that the metalbumin of Scherer is identical with pseudo-mucin, while paralbumin is a mixture of pseudo-mucin and albumin.

This work of Hammarsten's dislodged the ovarian fluids from the albuminous group and they were finally shown to be more or less closely allied to the mucins. When the mucins are considered it is found that there still exists some confusion, not only in the terms used for classification, but in the chemistry of the substances.

According to Mann,[6] Hammarsten has called a number of substances that closely resemble mucins in their composition and reaction by the name of mucoids. This separation from the mucins is to a large degree arbitrary. With a view of reserving the name mucin for the true mucinous substances secreted by the epithelium, Cohnheim calls all substances not derived from the epithelium, but from connective tissue, mucoids.

Numeister,[7] however, makes use of the term mucoid and gives as a particularly well-known example the material found in ovarian cysts, known as pseudo-mucin (metalbumin). This substance under all conditions is easily soluble in water and, in contra-distinction to the ordinary mucins, is not precipitated by acetic acid.

Mann,[8] in discussing pseudo-mucin, states that it very closely resembles the mucins but differs in that it is not precipitated by acidification with acetic or hydrochloric acids.

Also that nitric acid does not precipitate the solution, but renders it more opalescent and more viscous. It otherwise gives the reactions of true mucins. On another page[9] he says, concerning the substances found in ovarian tumors, that the mucoids are represented in these neoplasms in different forms. The most frequently found is the thick tenacious met- or par-albumin, sometimes known as pseudomucin, that is readily soluble in water. This substance is found especially both in the large glandular and in the proliferating papillary cysts. A less common form is a jelly-like mucoid that is insoluble in water, the so-called colloid. This fills the small glandular cystomata particularly, but is frequently found in the papillary cysts in connection with paralbumin.

This last-mentioned substance, colloid, was first described by Mitjukoff[10] in 1895 and given by her the name of paramucin. This material occurs in many ovarian cysts as a firm, trembling, clear yellow jelly that is insoluble in water, but which when treated with water containing hydrochloric acid undergoes skrinkage. It can be obtained as a white powder when acted upon by acid alcohol, and alcohol and ether. Small amounts of acetic acid precipitate it but in an excess of the acid it becomes soluble. When in an alkaline solution and warmed it is able to reduce copper.

Panzer,[11] in his article on ovarian colloid, says that in the human ovary there are found tumors consisting of a yellowish trembling colloid, known clinically as Cystoma pseudo-mucinosum, ovarian colloid, etc. The material in these tumors, known also as pseudo-mucin beta, para-mucin, has the following characteristics: It is not soluble in water. In a weak alkaline solution acetic acid causes a cloudiness with no flaky precipitate. If water be added to it when dry it will again swell up into colloid material. A reducing substance is also present, Fehling's solution although not immediately reduced by heating to the boiling point became clearly so after several minutes of continuous boiling.

Mann,[12] under the heading para-mucin, gives further important details. He states that it is insoluble in water, shrinks on the addition of acids and is changed by acid absolute alcohol into a fine non-hygroscopic powder, which is again converted into a jelly on being moistened with a little alkali solution. On the addition of large quantities of potassium or sodium hydrate para-mucin dissolves into a slimy solution that gives the ordinary mucin reactions: it is not coagulated by being boiled, and is not precipitated by ferrocyanic acid, although a slight turbidity may show itself. This probably depends upon traces of albumin being present. Tannic acid and lead acetate cause a precipitate, as do also acetic and mineral acids. In this last feature para-mucin resembles the true mucins and differs from pseudo-mucin. Excess of mineral acids renders the precipitate soluble.

Another substance belonging to the same group as those already mentioned, a " serosa mucin," is reported by Mann[13] as being found in a case of carcinoma of the stomach and peritoneum. In this the ascitic fluid was of yellow color, viscous and distinctly alkaline to litmus paper. A precipitate obtained on the addition of a one per cent solution of acetic acid was dissolved in just sufficient alkali, this was repeated three times. A neutral solution made up from this precipitate did not distinctly reduce a copper solution, but it did so after having been boiled for half an hour in a two per cent hydrochloric acid solution.

To briefly sum up we find that Scherer describes two bodies, metalbumin and paralbumin, as being found in ovarian cystomata. Hammarsten concludes that the characteristic appearances of the ovarian fluids are due to pseudo-mucin, this corresponding to Scherer's metalbumin. Paralbumin being pseudo-mucin plus albumin. Other investigators confirmed these findings and later Mitjukoff described a jelly-like material found in ovarian tumors to which she gave the name of para-mucin.

These different substances vary in their chemical characteristics from the true mucins and from each other so that the main divisions are mucin, pseudo-mucin, and para-mucin.

Wells[14] describes mucin, in its typical form, as being a compound proteid, consisting of a proteid radicle and a nitrogen-containing carbohydrate, glucosamin. This on being boiled with acid yields a substance that reduces Fehling's solution. It is acid in reaction, is dissolved in very weak alkali solutions and is precipitated by acetic acid. Pseudomucin is distinguished by being alkaline in reaction and by not being precipitated by acetic acid; para-mucin by yielding a reducing substance without previous decomposition by acids.

This incomplete discussion of the chemistry of the material found in these ovarian tumors leads up to the report of a case that was in the St. Louis Skin and Cancer Hospital in the service of Dr. George Gellhorn. I am also indebted to him for the following notes concerning the primary operation:

Mrs. L. H.; forty-five; white; February, 1907. Diagnosis is made of a large ovarian tumor extending upward half way between the umbilicus and ensiform process. Was operated upon in March, 1907, at which time nine and a half pounds of pseudo-mucinous masses were removed from the abdomen together with a ruptured cyst of the right ovary. This cyst is very thinned walled and contains a number of secondary cysts.

She was admitted to the hospital on Jan. 21, 1908, from which time she grew progressively worse till May 25, 1908, when she died. Shortly before this a complete perforation of the anterior abdominal wall had occurred and it was possible to obtain, ante-mortem, about one thousand cubic centimeters of material, by inserting a sterile glass tube. The substance obtained consisted of two portions, a thick fluid, which was slightly yellow in color, and mucous like in consistency, and many coagulated masses held in suspension.

This was collected in a sterile flask and portions subjected to various reagents in an attempt to determine its character. No bacteria were present, the fluid remaining sterile.

It was found that the material behaved as follows with the reagents mentioned: It was coagulated by boiling. Was precipitated by alcohol. Was coagulated by acetic acid, but did not redissolve on the addition of an excess of the acid. Was insoluble in water. Had not dissolved at the end of forty-eight hours. Dissolved in weak alkalies. Was precipitated from weak alkali solutions upon the addition of acetic acid. Did not reduce copper except on boiling in the presence of dilute hydrochloric acid. With nitric acid there was obtained a white precipitate which became yellow on boiling. On the addition of ammonia after the solution had become cool it changed in color to orange. From these reactions it would appear that the material found in this instance was more closely allied to the true mucins than to either pseudo- or para-mucin. From his brief description the material called serosa mucin by Mann would seem to have reacted rather similarly to this in question. Wells[15] states that true mucin is never present in ovarian cysts, yet this substance acts like mucin in that on being boiled with acid it yields a product that reduces Fehling's solution, it dissolves in very weak alkali solutions and is precipitated by the addition of acetic acid.

A question that might come up is whether or not the growth was ovarian in origin. Of that there seems little doubt. The operator who removed the tumor primarily made the diagnosis of a multilocular cyst of the ovary after removing it and the uterus. The subsequent history was that of a recurrence of the type that is common in such cases, and the autopsy confirmed the suspicions. At this time there was found a large irregular and nodular mass completely filling the lower half of the abdominal cavity. These nodules were covered by a dense membrane that was quite elastic to the touch and many, on opening, were found to contain large amounts of a lumpy gelatinous substance, others were filled with more liquid contents. In some of the cysts the material was quite clear, while in others it was cloudy and evidently infected. The colon bacillus had been recovered shortly before death from the fluid that escaped through the ulcerated area. The appearance was that of a multilocular cyst.

It would seem justifiable, therefore, to conclude that this particular case was one of a multilocular ovarian cyst in which was present a body more closely related to mucin than to pseudo- or para-mucin.

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THE JOURNAL OF MEDICAL RESEARCH is the direct continuation of "The Journal of the Boston Society of Medical Sciences." It is devoted to the prompt publication of original investigations in medicine; will be published at irregular intervals, as rapidly as material accumulates, and in numbers of irregular size. A volume will be limited to about five hundred pages, and when one is completed another will be begun.

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VOLUME XX., No. 2
(New Series, Vol. XV.)

FEBRUARY, 1909

Whole No. 112

THE JOURNAL OF MEDICAL RESEARCH

Continuation of the
JOURNAL OF THE BOSTON SOCIETY OF MEDICAL SCIENCES

EDITED BY
HAROLD C. ERNST, M.D.

EDITORIAL OFFICE: 240 LONGWOOD AVENUE
BOSTON, MASS., U.S.A.

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Journal of Medical Research.

(New Series, Volume XV.)

Vol. XX., No. 2. FEBRUARY, 1909. Whole No. 112.

EFFECT OF DILUTION UPON THE FLOCCULATION OF COLLOIDS.*

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As a sequel to some work done by us on the influence of temperature upon colloidal flocculation,[1] we have recently investigated the effect of flocculation upon dilution of the colloidal solutions or suspensions. So far as we can learn no systematic series of experiments in this direction have been published, so we may proceed at once to give our results without any previous discussion.

We will not attempt to describe the methods of making up the various colloidal solutions or suspensions, but may mention that distilled water was used throughout. Colloids, such as bacteria and some inorganic colloids, which necessarily contained some electrolytes when first made up, were subjected to dialysis for a week before use.

In the tables we give only the essentials, but for each systematic experiment one hundred tubes were used, there being twenty dilutions of the electrolyte and five dilutions of the colloid. Small tubes, three inches in length, were used and into each tube was pipetted one cubic centimeter of the colloid and one cubic centimeter of the electrolyte. The tubes were well shaken and allowed to remain at room temperature for twenty-four hours.

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* Received for publication Jan. 11, 1909.

I. Flocculation of colloids by electrolytes:

(*a.*) Acids.—If we take a typical colloidal suspension, such as mastic or bacteria, and treat it with hydrochloric acid, we find that dilution of the suspension has no effect whatever upon the flocculation limits. With considerable dilution of the colloid the flocculation may become less complete, but the limits remain the same. Beyond a certain point in the dilution of the colloid there is no visible precipitation.

Table I. shows the results obtained with mastic and Staphylococcus pyogenes aureus. In the latter case there is a well-defined prezone, since with HCl at normal there is no flocculation. Such a prezone is characteristic of all bacteria when treated with acids. In Table II. are given experiments with two inorganic colloidal solutions: basic ferric hydroxide and acid arsenic sulphide. The results are the same as in the case of the suspensions.

TABLE I.

Hydrochloric acid with colloidal suspensions in varying dilutions.

HCl.	Mastic — Dilutions of "Orig."					HCl.	Staph. Pyogenes Aureus — Dilutions of "Orig."				
Final Dilutions.	1/10	1/60	1/120	1/200	1/300	Final Dilutions.	1/20	1/60	1/120	1/240	1/320
Normal	+++	+++	+++	+++	+	Normal	—	—	—	—	—
N. 1/10	+++	+++	+++	+++	+	N. 1/10	++	+	+++	+	+
1/100	+++	+++	+++	+++	+	1/100–1/400	+++	+++	+++	+	+
1/200	+	+++	+++	+++	+	1/1000	+	+	+	+	Trace.
1/400	—	—	—	—	—	1/2000	—	—	—	—	—

"Orig." = Original dilution made up very thick.

TABLE II.

Hydrochloric acid with colloidal solutions in varying dilutions.

HCl.	Ferric Hydroxide — Dilutions of "Orig."					HCl.	Arsenic Sulphide — Dilutions of "Orig."			
Final Dilutions.	1/20	1/40	1/80	1/120	1/200	Final Dilutions.	1/10	1/40	1/60	1/120
Normal	+++	+++	+++	+++	+++	Normal	+++	+++	+++	+++
N. 1/4	+++	+++	+++	+++	+++	N. 1/10	+++	+++	+++	+++
1/10	—	—	—	—	—	1/40	+	+	+	+
						1/80	—	—	—	—

We have made systematic tests with fifteen different colloids against hydrochloric acid in addition to a few against HNO_3 and H_2SO_4, with essentially similar results throughout. In a few instances, however, there is no flocculation at all, *e.g.*, agar and silicic acid.

(*b.*) Salts. — Tables III., IV., and V. are illustrative of results obtained with mono- and divalent salts. It makes no difference if the colloids are inorganic, organic, or organized, positive or negative, their dilution has no appreciable effect upon their flocculation limits by salts of alkalis, alkaline earths, or of heavy metals.

TABLE III.

Flocculation by monovalent salts.

NaCl.	Ferric — Hydroxide "Orig."			KCl.	Colloidal — Gold "Orig."			AgNO$_3$.	Mastic — "Orig."		
Final Dilutions.	1/20	1/80	1/200	Final Dilutions.	1/2	1/4	1/8	Final Dilutions.	1/10	1/60	1/240
Normal	+++	+++	+++	Normal.......	++	+	+	Normal.......	+++	+++	+++
" 1/10....	+++	+++	+++	" 1/10...	++	+	+	" 1/2....	+++	+++	+++
" 1/50....	+++	+++	+++	" 1/20...	++	+	+	" 1/10...	+++	+++	+++
" 1/100...	—	+++	+++	" 1/40...	++	Trace.	—	" 1/40...	—	—	—
" 1/200...	—	—	—	" 1/80...	—	—	—				

TABLE IV.

Flocculation of colloids by alkaline earths.

CaCl$_2$.	Ferric Hydroxide — Dilutions of "Orig."					CaCl$_2$.	Coli Agglutinin * Bacilli — Dilutions of "Orig."			
Final Dilutions.	1/20	1/40	1/80	1/120	1/200	Final Dilutions.	1/100	1/200	1/400	1/800
Normal...... to	+++	+++	+++	+++	+++	Normal....... to	++	++	++	++
N. 1/50......	+++	+++	+++	+++	+++	N. 1/10,000...	++	++	++	++
1/100.....	—	—	++	++	+++	1/12,000...	—	—	—	—
1/200.....	—	—	—	—	—					

* Agglutinin Bacteria. Dialyzed bacteria treated with dialyzed immune serum and washed free of serum. Treated in this way the bacteria remain in suspension, but are very susceptible to flocculation by electrolytes.

TABLE V.

Flocculation of colloids by salts of heavy metals.

$CuCl_2$.	Arsenic Sulphide — Dilutions of "Orig."				$ZnSO_4$.	Bac. Pyocyaneus — Dilutions of "Orig."			
Final Dilutions.	1/10	1/40	1/60	1/120	Final Dilutions.	1/10	1/60	1/120	1/240
Normal............	+++	+++	+++	+++	Normal	++	++	++	++
to N. 1/400	+++	+++	+++	+++	to N. 1/200	++	++	++	++
1/600	—	+	+++	+++	1/400.........	+	+	+	Trace.
1/800	—	—	—	—	1/1000........	+	Trace.	Trace.	—

We find, then, that in flocculation of colloids by electrolytes, excluding for the present the trivalent salts, there is a definite type of flocculation which occurs when the colloids are used in varying dilutions. Certain irregularities may be noted in some cases, but the irregularities are not sufficient appreciably to modify the general type which may be expressed diagrammatically as a horizontal tube.

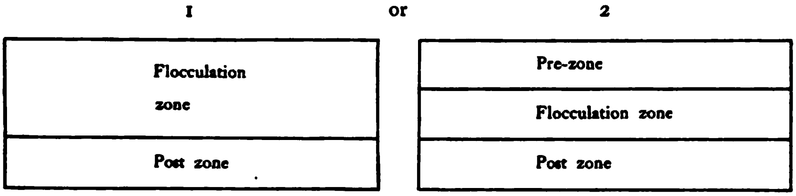


Diagram 2, showing the prezone, is a modification of the general type which occurs with bacteria; constantly when treated with an acid, and occasionally with a divalent salt of a heavy metal.

We may conclude from these observations that in flocculation of colloids by electrolytes, exclusive of trivalent salts, the flocculation limits are not in any way dependent upon the relative concentrations of the electrolyte and colloid.

We have not tested every colloid (16) with every electrolyte (10) used, but in about fifty systematic tests we have invariably obtained the same type of flocculation, the irregularities in no case being greater than in some of the tables given above.

II. Flocculation of colloids by colloids of opposite sign (+ and — colloids):

In this series of experiments we find the prevailing conditions are entirely different from those obtaining in flocculation by electrolytes. Very definite zones of flocculation are encountered, the position of the zones in the tables being dependent upon the relative concentrations of the two colloids.

TABLE VI.

Flocculation of colloid by colloid. Ferric hydroxide with mastic and bacteria.

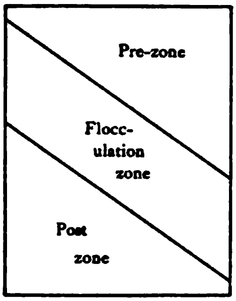
+ Colloid.	Mastic — Dilutions of "Orig." — Colloid.					+ Colloid.	Bac. Coli Communis — Dilutions of "Orig." — Colloid.				
Fe(OH)$_3$ Final Dilutions of "Orig."	1/10	1/60	1/120	1/200	1/300	Fe(OH)$_3$ Final Dilutions of "Orig."	1/20	1/60	1/120	1/240	1/320
1/10 .... to	—	—	—	—	—	1/50	—	—	—	—	—
1/500	—	—	—	—	—	1/150	+++	—	—	—	—
1/1000	+++	—	—	—	—	1/350	—	+++	—	—	—
1/2000	+++	+++	—	—	—	1/500	—	+++	—	—	—
1/3000	+++	+++	+++	—	—	1/800	—	+	+++	—	—
1/5000	—	+++	+++	+++	+	1/1000	—	—	+++	—	—
1/8000	—	—	+++	+++	++	1/1500	—	—	—	+++	+++
1/16,000	—	—	—	Trace.	Trace.	1/2000	—	—	—	+++	+++
1/20,000	—	—	—	—	—	1/3000	—	—	—	—	—

TABLE VII.

Flocculation of colloid by colloia.

+ Colloid. Fe(OH)$_3$.	Silicic Acid — Dilutions of "Orig." — Colloid.					+ Colloid. Al(OH)$_3$.	Staphylococcus Pyogenes Aureus — Dilutions of "Orig." — Colloid.				
Final Dilutions of "Orig."	1/50	1/100	1/200	1/400	1/800	Final Dilutions of "Orig."	1/20	1/60	1/120	1/240	1/320
1/50	+	—	—	—	—	1/100	+++	—	—	—	—
1/100	++	+	—	—	—	1/200	—	+++	—	—	—
1/600	++	++	++	+	—	1/300	—	+++	—	—	—
1/1000	—	++	++	++	—	1/400	—	—	+++	—	—
1/1500	—	—	++	++	+	1/600	—	—	—	+++	—
1/2000	—	—	—	++	+	1/800	—	—	—	+++	++
1/4000	—	—	—	—	+	1/1000	—	—	—	—	++
1/5000	—	—	—	—	—	1/1500	—	—	—	—	—

The type of flocculation of colloids by colloids may therefore be expressed by the diagram:



III. Flocculation of colloids by salts of trivalent metals with an explanation of the phenomenon of irregular series (Unregelmassige Reihen of Bechhold[2]).

The phenomenon of irregular series in flocculation, as Bechhold originally expressed it, is now very well recognized and is specially marked in flocculation by trivalent salts. Bechhold suggests that the hydrolytic dissociation and consequent presence of basic hydroxides may have some influence in the causation of irregular series, the hydroxide acting as a protective colloid at certain dilutions. Our experiments have shown that Bechhold was perfectly correct in his surmise as to the influence of the hydrolysis, but the hydroxide, so far from acting as a protective colloid, is itself an active factor in the flocculation.

We append first a table condensed from a previous article[3] to show the principle involved:

TABLE VIII.

Illustration of irregular series.

$AlCl_3$.	Mastic.	Colloidal Platinum.	$FeCl_3$.	Mastic.	Coli Agglutinin Bac.	Typhoid Agglutinin Bac.
Normal	+++	+++	Normal	+++	—	—
N. 1/2	+++	+++	N. 1/100	—	+++	+++
1/20	—	+++	" 1/1000	—	—	—
1/200	—	—	" 1/2000	—	+++	—
1/2000	+++	+++	" 1/4000	+++	+	+++
1/20,000	—	—	" 1/8000	—	—	—

Let us now test the effect of dilution of the colloid upon the phenomenon of irregular series.

TABLE IX.

Mastic treated with ferric and aluminium chloride.

Ferric Chloride.	Mastic — Dilutions of "Orig."					Aluminium Chloride.	Mastic — Dilutions of "Orig."				
Final Dilutions.	1/10	1/60	1/120	1/200	1/300	Final Dilutions.	1/10	1/30	1/60	1/120	1/300
Normal	+++	++	+	+	+	Normal	+++	+++	+++	+++	+
" 1/20........	+++	++	+	+	+	N. 1/2..........	+++	+++	+++	+++	+
" 1/40–1/500..	—	—	—	—	—	N. 1/10–1/600...	—	—	—	—	—
" 1/1000......	+++	—	—	—	—	1/1000.......	++	—	—	—	—
" 1/2000......	++	++	—	—	—	1/2000.......	+++	+++	—	—	—
" 1/3000......	—	+++	—	—	—	1/3000.......	+++	+++	++	—	—
" 1/4000......	—	+++	++	—	—	1/4000.......	+++	+++	+++	+++	—
" 1/6000......	—	++	+++	++	—	1/6000.......	—	++	+++	+++	+++
" 1/8000......	—	—	+	++	+	1/10,000.....	—	+++	+++	+++	+++
" 1/10,000....	—	—	—	—	+	1/20,000.....	—	—	—	+++	+++

TABLE X.

Bacteria and colloidal platinum treated with ferric chloride.

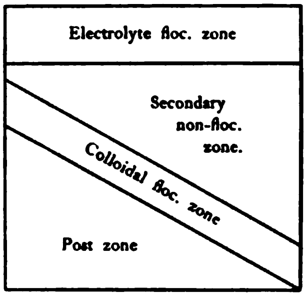
Ferric Chloride.	Bac. Coli Communis — Dilutions of "Orig."					Ferric Chloride.	Colloidal Platinum —Dilutions of "Orig."		
Final Dilutions.	1/20	1/60	1/120	1/240	1/320	Final Dilutions.	1/2	1/8	1/20
Normal	+	+	—	—	—	Normal	+++	+++	+
N. 1/20–1/200	+++	+++	++	+	+	N. 1/10	+++	+++	+
1/300	+++	+++	+	+	+	1/100	+++	+++	+
1/400	+++	+++	+	—	—	1/500 to	—	—	—
1/600	++	+++	+++	—	—	1/5000	—	—	—
1/1000	—	++	+++	—	—	1/6000	+++	—	—
1/1500	—	—	+++	++	+	1/10,000	+++	—	—
1/2000	—	—	+	++	++	1/12,000	+	+++	—
1/3000	—	—	—	+	++	1/20,000	—	+++	+
1/4000	—	—	—	—	+	1/30,000	—	—	+

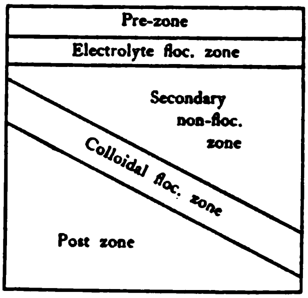
On examination of these two tables, IX. and X., we find that in each case the upper zone of flocculation has the type of flocculation by electrolytes, whereas the type of the lower zone corresponds to that of flocculation between two colloids. There seems to be every reason for supposing that the lower flocculation zone is due to the ferric or aluminium hydroxide formed by hydrolytic dissociation, while the upper zone is caused by the electrolyte ions present, probably chiefly by the H' ions of the hydrochloric acid, the other product of the hydrolysis, which proceeds according to the equation $FeCl_3 + 3H_2O = Fe(OH)_3 + 3HCl$.

In the case of B. coli communis the two zones are fused at first but at higher dilutions of the suspension separate out, each zone developing the characteristic appearance which with mastic and colloidal platinum is evident from the beginning. With B. coli communis also we encounter the prezone with which we have already become familiar in flocculation of bacteria by acids. Neither mastic nor colloidal platinum show such a prezone with acids, nor do we find any evidence of it in their upper zones when they are flocculated by salts of trivalent metals.

The general type of flocculation by salts of trivalent metals may be expressed by the diagram:

1 or 2





Results with salts of trivalent metals, however, do not always come out so clean cut and obvious as in the two

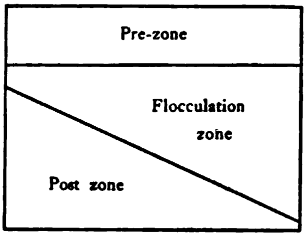
Tables IX. and X. given above. The following table of agar with ferric chloride and Staphylococcus pyogenes aureus with aluminium chloride shows another type with which we have occasionally met. It may be noted that HCl and other mono- and divalent electrolytes do not flocculate agar at all, but they flocculate Staphylococcus pyogenes aureus in the usual way.

TABLE XI.

Angular type of flocculation.

$FeCl_3$ Final Dilutions.	Agar Dilutions Per Cent.					$AlCl_3$ Final Dilutions.	Staphylococcus Pyogenes Aureus.				
	1/20	1/60	1/100	1/200	1/400		1/20	1/60	1/120	1/240	1/320
Normal	—	—	—	—	—	Normal	—	—	—	—	—
N. 1/10	—	—	—	—	—	N. 1/4	—	—	—	—	—
" 1/100	—	—	—	—	—	" 1/20–1/300	+++	+++	+++	+	+
" 1/200	?+	+	+++	+++	+++	" 1/400	+++	+++	+++	+	+
" 1/400	?+	?+	+++	+++	+++	" 1/800	—	+	+++	+	+
" 1/800	?+	?+	+	+	+	" 1/1000	—	—	+++	+	+
" 1/1000	—	—	Trace.	+	—	" 1/1500	—	—	—	+	+
" 1/1200	—	—	—	—	+	" 1/2000	—	—	—	—	Trace.
" 1/2000	—	—	—	—	Trace.	" 1/3000	—	—	—	—	Trace.

In such instances we find that the lower limits of the flocculation descend in the usual way, but there is no separation from an upper zone and we get a type which forms an angle of about 45°.



The lower limits of the flocculation zone are obviously determined by the hydroxide, but the upper limits seem to be interfered with in some way so that atypical results are obtained. When the salt of the trivalent metal is highly diluted there are practically only HCl ions and hydroxide present, but at higher concentrations a variety of intermediate products are formed, the presence of which may introduce disturbing factors in some instances. We do not think that the occasional occurrence of the angular type disproves the rule laid down for Tables IX. and X.

That the presence of electrolytes may in some cases have influence upon the type of flocculation is shown in Table XII., in which we find that if the colloids are made up with physiological salt solution instead of distilled water the normal colloidal type is transformed to the angular type of flocculation. In this case when NaCl is present, an excess of laked red cells is able to hold the ferric hydroxide in solution, but an excess of ferric hydroxide cannot hold the laked cells in solution. Now laked cells of themselves are not flocculated at all by NaCl, but a reference to Table III. shows that ferric hydroxide is very susceptible to the action of NaCl.

Table XII.

Influence of .9 per cent. salt solution upon the flocculation type.

$Fe(OH)_3$ in Distilled Water.	Laked Red Cells in Distilled Water.					$Fe(OH)_3$ in Distilled Water.	Laked Red Cells in 1.8 Per Cent Salt Solution.				
	1/20	1/60	1/120	1/240	1/400		1/4	1/12	1/24	1/50	1/100
1/100.........	++	+	—	—	—	1/50.........	++	++	++	++	+++
1/300.........	—	+++	—	—	—	1/100........	++	++	++	++	+++
1/500.........	—	+	+++	—	—	1/200........	+	++	++	++	++
1/1000........	—	—	+++	—	—	1/400........	—	+	+	++	++
1/2000........	—	—	—	+++	—	1/700........	—	—	—	+	+
1/3000........	—	—	—	+++	++	1/800.... ...	—	—	—	—	+
1/6000........	—	—	—	—	++	1/1000.......	—	—	—	—	—

Flocculation of bacteria by immune serum:

On testing various bacteria, Typhoid, Paratyphoid, Pyocyaneus and Coli communis with their respective immune serums, we find that the colloidal type of flocculation is followed; the lower limits of flocculation descending with increased dilution of the bacilli. In early stages of the experiment some indications of a prezone are occasionally evident, but in twenty-four hours the prezone has entirely disappeared so that we get the angular type of flocculation. Table XIII. illustrates the action of immune serum upon Coli communis and Paratyphoid.

It seems at first sight somewhat paradoxical to find that if bacteria are loaded with agglutinin (agglutinin bacteria), and then treated with an electrolyte, they follow the electrolyte type of flocculation (Table IV.), whereas if the same bacteria are treated in the ordinary way with immune serum the colloidal type is followed, but it must be remembered that in the first case each bacillus is equally loaded, or approximately so, with agglutinin, and that therefore all are rendered equally unstable in the presence of an electrolyte, while in direct treatment with immune serum, as shown in Table XIII., the agglutinin has a different ratio of distribution between the bacilli at each different dilution of the latter.

TABLE XIII.

Flocculation of bacteria by immune serum.

Immune Serum.	Bac. Coli Communis — Dilutions of "Orig."					Immune Serum.	Bac. Paratyphosus — Dilutions of "Orig."				
Final Dilutions.	1/2	1/8	1/30	1/60	1/120	Final Dilutions.	1/2	1/8	1/24	1/60	1/120
1/20	++	+++	+++	+++	+++	1/2	+++	+++	+++	+++	+++
1/200	++	++	+++	+++	+++	1/20	++	+++	+++	+++	+++
1/2000	+	++	++	++	+++	1/200	++	++	++	++	++
1/10,000	—	+	++	++	++	1/2000	—	++	++	++	++
1/20,000	—	—	+	+	+	1/4000	—	—	+	++	++
1/40,000	—	—	—	+	+	1/8000	—	—	—	+	+
1/50,000	—	—	—	—	+	1/10,000	—	—	—	—	+

The angular colloidal type of flocculation is also apparent on testing for group reactions (Table XIV.).

TABLE XIV.

Group reactions.

Coli Immune Serum.	Typhoid Bacilli — Dilutions of "Orig."				Paratyphoid Bacilli — Dilutions of "Orig."				
	1/2	1/10	1/40	1/100	1/4	1/10	1/30	1/60	1/120
1/4	++	++	+++	+++	++	+++	+++	+++	+++
1/20	+	+	+	+	+	++	++	+++	+++
1/40	−	+	+	+	−	+	++	++	+++
1/80	−	−	+	+	−	−	+	++	++
1/120	−	−	+	+	−	−	−	+	+
1/160	−	−	+	+	−	−	−	−	+
1/200	−	−	−	+	−	−	−	−	−

CONCLUSIONS.

1. We find as a result of these observations that there are two different types of flocculation of colloids:

   a. The electrolyte type. On flocculation of colloids by acids or salts of mono- or divalent metals, dilution of the colloid has no effect upon the flocculation limits. There is no relation between the concentration of the electrolyte and the concentration of the colloid.

   b. The colloidal type. On flocculation of a colloid by a colloid of opposite sign the flocculation limits are dependent upon the relative concentrations of the two colloids. If either colloid is much in excess there is no flocculation.

2. The trivalent salt type. With salts of trivalent metals, on account of hydrolytic dissociation, there is a combination of the electrolyte type and the colloidal type, leading to the phenomenon of irregular series. The upper zone of flocculation conforms to the electrolyte type, and the lower zone to the colloidal type.

3. Agglutination of bacteria by immune serum follows

the colloidal type of flocculation, although an excess of immune serum does not necessarily inhibit flocculation.

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ENTEROKINASE IN INFANCY.*

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Our early knowledge of enterokinase is obtained from Pawlow[11] who used intestinal secretion in all cases instead of extracts of the alimentary tract. Ellinger and Cohn,[6] however, used extracts of human jejunum, and found them active. Otto Cohnheim,[3] in his work on "The Physiology of Digestion and Nutrition" (page 39), says that enterokinase is a specific product of the epithelial cells of the small intestine and is only produced by them when the pancreatic juice enters the intestine. Furthermore, he states (on page 120) that the enterokinase is soluble in dilute alcohol and will endure heating to 70° C. without harm, and since it is generally regarded as an enzyme it will not withstand boiling temperature. Bayliss and Starling[1] state that no substance but this activator can convert trypsinogen into active trypsin, that it is secreted chiefly in the upper part of the small intestine and that it is decomposed at a temperature of 40° C. Cohnheim[4] has further shown that an excess of intestinal extract can hinder the action of trypsin, in fact can stop it and cause an already active tryptic digestion to cease. Unfortunately, access to his original work could not be had and the review (in the *Jahresbericht für Thierchemie*) did not give the exact amount of the intestinal extract which produced the most active tryptic digestion. Hallion and Carrion[7] consider since the duodenum, the source of the production of the enterokinase, is much oftener affected than the pancreas, insufficient intestinal digestion is oftener due to the lack of the former than of trypsin. Carmelo Ciaccio[2] found enterokinase in the spleen and lymph glands in which it was very much increased during infections. I could find

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* Presented before the American Society of Biological Chemists at the annual meeting at Baltimore, Md., Dec. 28, 1908. Received for publication Jan. 11, 1909.

no reference to its increase or diminution in the duodenum its natural site, during infections.

Hamburger and Hekma[8] had an opportunity during the persistence of an intestinal fistula in a man to observe the intestinal secretion for a period of sixty-six days and came to the conclusion that enterokinase could not activate an unlimited amount of trypsinogen as Bayliss and Starling had contended; in fact that the combination of the two must take place within very narrow limits. From this they concluded that the activator was not a true ferment. Furthermore, they showed that it was not identical with erepsin which was destroyed at a temperature of 59° C. after two hours heating, while the kinase required three hours heating at 67° C. for its destruction. My original intention in this investigation was to prove or disprove the statement of Hallion and Carrion that the secretion of enterokinase was oftener deficient in intestinal disturbances than that of trypsin.

The only way open to me for study of this question was by means of extracts of human organs obtained at autopsy, since Cohnheim and others had found that very active digestive agents could be procured from these, perhaps not much lacking in efficiency when compared with results obtained from the true intestinal juices. My original intention was only partially carried out, for the work was begun in winter when intestinal disturbances in infants are much less common, but as so little is known about the presence of this coferment in infancy, it seemed worth while to examine organs of a number of children to determine at what age it appears and also some of the conditions pertaining to its extraction from infant organs. As the continuation of the work shows most of these individuals died from pneumonia, what effect this disease may have had upon the presence and activity of enterokinase cannot, of course, be stated.

At the autopsy the pancreas and duodenum were carefully separated from each other, wrapped individually in gauze and brought to the laboratory in as fresh condition as possible. (My earnest thanks are due to Professor Leary of Tufts College for his aid in procuring these specimens.) Arrived at the laboratory, they were carefully minced by an Enterprise meat cutter, washed by running water until free from blood, since the latter, as is well known, has a tendency to inhibit tryptic digestion, and after the finely divided material was almost white, placed in glycerine; the duodenum in ten cubic centimeters of the extractive and the pancreas in thirty, where they were allowed to remain twenty-four hours. After this period they were strained through gauze and if the extract was not sufficient to equal ten and thirty cubic centimeters, respectively, the tissue was washed with enough glycerine to make up those amounts. It will be well to state at this point that C. Delezenne[5] claims that blood serum does not affect the action of trypsin, but, in even small amounts, does hinder the activation of the trypsinogen; in other words, possesses an energetic antikinase; in either case the washing of the tissue was justified. Physiological salt solution was used at first, but was found much less effective than the glycerine. Usually the extracts were of a pale straw color, though often slightly blood stained when the washing was insufficient. On account of the inability of salt solution to extract the ferment, it is not probable that much of the enzymes was lost by washing with water.

It was now necessary to select a method for the determination of the digestive power of the ferment extracted; a method was desired which would enable me to compare the organs of the different individuals with fair accuracy, yet not one requiring enormous expenditure of time. Such an one had been devised by Loehlein[10] for quantitative trypsin determination, a method first used for pepsin determination by Vohlhard, which only required that the addition of hydrochloric acid be deferred until the digestion was completed. The principle of this procedure, as is well known, depends upon the fact that the acid combines both with the undigested protein as well as with the digested proteose, but the latter passes through the filter paper when the former is precipitated by sodium sulphate, and the acidity of the filtrate when measured by tenth normal sodium hydrate is increased by every increase in the digested protein (or proteose) and hence in trypsin. Therefore, for comparative purposes the number of cubic centimeters of the sodium solution represents the amount of digestive enzyme present.

The only objection to this method that at once appears to one is whether the degree of alkalinity is sufficient for the energetic proteolytic power of the trypsin; this can be answered by the experience of Kantiz,[12] who found a solution containing from one-seventieth to one two-hundredth normal hydroxyl ions to be the best suited for this purpose; the solution recommended by Loehlein, however, contains one eighty-fifth normal hydroxyl ions and hence is well within the limit.

At first the casein gave some trouble in determining the end reaction with the filtrate because it contained some fat which gave a yellow color that obscured the change from a colorless to a pink solution. The casein was extracted several times with ether and then dried, whereupon a perfectly colorless solution ensued. Phenolphthalein was always used for an indicator.

The points to be first established were whether the stock solution would undergo an autodigestion; whether the pancreatic extract had any digestive power or not; whether this power was magnified by the addition of intestinal extract, and finally if the combination of pancreatic and intestinal extract were actually the cause of any increase of digestion which might occur beyond that taking place in the stock solution itself. Hence four digestions were prepared; the first consisting of one hundred cubic centimeters of the stock solution alone, diluted to three hundred; the second of one hundred of the stock solution, ten cubic centimeters of pancreatic extract and water to three hundred; the third of the same amount of casein solution, the same amount of pancreatic extract and five cubic centimeters of intestinal extract with water to three hundred; the fourth of exactly the same ingredients and proportion except that the extracts were cooked in a test-tube placed in a water bath which was kept briskly boiling for thirty minutes. This certainly will destroy any trypsin present and should render any kinase inert, if it partakes of the nature of an enzyme as it is supposed to do. All digestions were saturated with chloroform and a layer of toluol poured over them to avoid any possible suspicion of bacterial action, and I may say that, as far as odor may inform one of such action, these digestions remained bacteria free. They were all placed in a brood oven at a temperature of 37° C., where they remained twenty-four hours. After the digestion was completed, in pursuance of the method of Loehlein, eleven cubic centimeters of normal hydrochloric acid were added and a saturated solution of sodium sulphate until the total reached four hundred cubic centimeters. A clear and colorless filtrate always resulted, except as stated above, upon filtration, two hundred cubic centimeters of which were always titrated with tenth normal sodium hydrate and the double of this factor (equal to the proteose in four hundred) used as the factor in the table which follows. Under remarks is stated the time elapsing after death when the autopsy was performed, the age of the child and the disease from which it died.

No.	Casein alone.	Pancreatic alone.	Amount Digested.	Pancreatic and Intestinal.	Amount Digested.	Pancreatic and Intestinal Cooked.	Amount Digested.	Remarks.
1	56.8	75	38.2	106	49.2	58.8	2	9 months old. Pneumonia.
2	57.2	106	48.8	110	52.8	64	6.8	18 months old. 24 hours P.M.
3	59.6	96	36.4	96.4	36.8	66	6.4	2 months old. 3 hours P.M.
4	59.6	107.2	47.6	105.4	45.8	69.6	10	1.5 months old. 6 hours P.M.
5	56.8	102.2	45.4	104.8	48	72.2	15.4	3 months old. 5 hours P.M.
6	55.8	69.8	14	85.4	28.36	55.7	1	4 months old. 14 hours P.M.
7	57.2	130.4	73.2	134.4	77.2	58.2	2	3 months old. 5 hours P.M.
8	54.6	101	46.4	101.6	47	57.9	3.2	11 months old. 5 hours P.M.
9	58.6	82.4	23.8	92	33.4	63.9	5.2	12 months old. 15 hours P.M.

All of these children died of pneumonia with the exception of number two, of enteritis; number six and seven, of empyema; number eight, of heart disease, and number nine, of rachitis.

The first thing that attracts our attention is that the pancreatic extract alone contains apparently an active trypsin as shown by the marked increase of the digestive product with that extract over that of the casein solution alone; as the latter was subjected to exactly the same conditions of temperature and underwent the same manipulations as the former, no other deduction is permissible. That the ferment in the glycerine was in its active form is very doubtful unless an active agent is found in the pancreas which according to the authorities quoted is very doubtful. How then did it become activated? We have several possibilities to consider: first, there is the statement of Hammersten[13] that pancreas or an infusion of it has the proferment converted to the ferment by the action of air and water; further, there is the bare chance that the pancreas and the intestine were brought in contact with each other at the autopsy, which is very doubtful on account of the care employed to avoid this at my request; again, it is very reasonable to suppose that the alkali used for the solution of the casein when brought in contact with the proferment may render it active. The first view is probably the correct one, since active trypsin extracts of the pancreas were made long before the kinase was ever known.

Upon the addition of the intestinal extract it is noticed that in many instances, not in all, there is a marked increase in the amount of proteoses as measured by the alkali used. For instance, in number one there is an increase of eleven cubic centimeters while the control shows only an increase of two over the casein alone. Number six also shows an excess of fourteen while the control shows only one; number seven gives four, but as the control has a digestion of two this result is too near the limit of error to place any dependence upon it.

In two instances the strong evidence of digestion with cooked extracts is inexplicable; all the extracts were subjected to the same temperature, at least 98° C., as shown by an inserted thermometer and for the same period, thirty minutes, and all digestive action should have been destroyed. Since the degree of error does not approach, except in two instances, the increase of the results from the mixed extracts over the pancreas alone, I think we may conclude that this shows the presence of a kinase. Age apparently has no effect upon the results; as good a digestion was obtained with the organs of an infant of one and a half months old as one eighteen, while both are surpassed by one of three months. No distinctive effect can be seen either from variations of the time elapsing between death and the autopsy, at least within twenty-four hours; two equally good results were obtained with organs removed six and twenty-four hours after death.

As these results were not as distinctive as could be wished, it was decided to prepare another series in which the intestinal extract alone should be cooked; if kinase were present and is destroyed by heat, we would have more emphatic evidence of its action in the digestion with the double extracts. Hence the same routine was followed except that the intestinal extract was cooked thirty minutes and after cooling added to the mixture which contained the casein and the pancreatic extract. This formed one control, while the casein solution alone formed the other. It was found necessary to use the latter in every case since the casein solution differed in its titer from one period to another, while the specimen which had remained in the brood oven for twenty-four hours differed in its titer from the original stock solution in that the former always required one or two more cubic centimeters of the alkali than the latter; this can only be explained by the supposition that an autolytic process was going on, though this was avoided as much as possible by cooking the stock solution for a moment when it was made.

COOKED INTESTINAL EXTRACT AS CONTROL.

No.	Casein alone.	Pancreatic alone.	Amount Digested.	Pancreatic and Intestinal.	Amount Digested.	Pancreatic and Intestinal Cooked.	Amount Digested.	Remarks.
10	58.2	121.4	63.2	120.4	62.2	128.4	70.2	12 months old. 8 hours P.M.
11	59.6	89.4	29.8	120	60.4	90.8	31.2	14 months old. 24 hours P.M.
12	59.6	103.8	42.2	107.6	48	105.6	46	5 months old. 8 hours P.M.
13	56.2	141.2	85	140	83.8	140	83.8	11 years old. 2 hours P.M.
14	56.4	72.2	15.8	115.4	59	72	15.6	4 months old. 10 hours P.M.

Three of these children died of pneumonia, numbers eleven, twelve, and fourteen; number ten died of rachitis and number thirteen of pericarditis. Yet no distinction based upon the character of the disease can be discovered.

Upon examination of the figures, it is readily seen that eleven and fourteen show a marked increase of the digestive products with the combined extracts over that of the pancreas alone, while twelve has less increase, but when compared with the cooked intestinal extract still shows evidence of the effect of a kinase. How otherwise are we to interpret these results than that there is some agency which activates the pancreatic extract, in other words, a true kinase obtained from the intestine? Now when this latter is heated, we would expect to obtain the same results as with the pancreas alone. If we note the column containing these figures, we shall find that this expectation is fulfilled. In the first case there is apparently a marked discrepancy, but as this is so entirely unlike the others it must be regarded as a mistake of technic.

As before, no law of amount can be based either upon the age of the child or upon the time of the autopsy after death. Equally large quantities of kinase can be found in infants of four months as in those of fourteen, while almost as much of this substance is found at an autopsy ten hours after death as one twenty-four hours.

The increase of digestive power of the child of eleven years, however, is noticeable; whether this is only relative or absolute I cannot say; unfortunately the organs were not weighed, so that no comparison can be made between the actual amount of tissue and its digestive power.

Another point remained to be investigated before we could accept these results as absolutely demonstrating the presence of a kinase; could not the increase of digestion noted when intestinal extract was added be due to proteolytic power of the intestinal secretion itself? True, all authorities are generally agreed that this secretion possesses no power to modify proteins nor fats, but it is still possible that the erepsin may act upon the casein, the only protein which it will affect; hence it was thought advisable to repeat these experiments with this variation, that the control contain cooked pancreatic extract added to uncooked intestinal extract; other than this modification, the digestions were of the same character as before. In this way it is very easy to demonstrate whether the intestinal extract has digestive power per se.

Cooked Pancreatic Extract as Control.

No.	Casein alone.	Pancreatic alone.	Amount Digested.	Pancreatic and Intestinal.	Amount Digested.	Pancreatic and Intestinal Cooked.	Amount Digested.	Remarks.
15	60	91.2	30.8	97	36.6	80.6	20.2	24 days old. 24 hours P.M.
16	55	86	31	113.6	58.6	54	0	2 years old. 24 hours P.M.
17	53.4	91	37.6	92.2	38.8	54	.6	7 months old. 24 hours P.M.
18	51.8	72.8	21	85	33.2	51	0	15 months old. 6 hours P.M.

In this list, numbers fifteen and seventeen died of pneumonia, sixteen of measles, and eighteen of empyema. As can be seen, no significance can be attached to the cause of death as affecting the digestive power of the extracts. We have marked evidence of a kinase in three instances bearing in its amount no relation to the age of the infant nor to the time of the autopsy elapsing after death. That this increased power of the tryptic digestion is due to the kinase and not to a proteolytic action of a possible erepsin in the casein is readily shown by the fact that the intestinal extract when mixed with cooked pancreatic extract exhibits no action whatever; in only one instance is this not true, in the first where we are obliged to account for this apparent action in some other way since it is so contrary to the other three.

Of the antikinase which has been mentioned by Cohnheim and others, I have seen no evidence; if it were present, then the pancreatic digestions alone should have accomplished more than the mixed digestion. Upon a perusal of these figures no instance of that nature can be found. It is of course possible that with smaller portions of the intestinal extract better results might have been obtained, but at least there is no case in which the antikinase was able to nullify the effect of the pancreas infusion. Whether in the instances where there was no increase of the action of the two extracts over that of the pancreas alone, antikinase played any part cannot of course be told; the presumption is rather that there was no kinase under these conditions. It is interesting to note also that as early as twenty-four days after birth there was present both trypsin in the pancreas and probably a kinase in the intestine, though the evidence of the latter is not as good as could be wished.

Of these eighteen children we may say, with all fairness, that seven possessed intestines in which kinase was being secreted while two more had fair but less absolute evidence of the possession of the same ferment.

In closing, based upon this work, we may claim that the following conclusions are justifiable:

(1.) That from the intestines of some infants, not all, from twenty-four days of age on, a kinase can be isolated which follows all the conditions laid down for that substance when present in the natural intestinal secretion.

(2.) That the disease from which the child dies (apart from intestinal disturbances which I have not yet had the opportunity to study), the age of the child and the period of the autopsy after death do not modify the amount of this coferment in any way which I have been able to discover.

(3.) As far as these experiments go, no evidence of an antikinase was discovered. In every case the digestion with pancreas and intestinal extract never produced less digestive products than the pancreas alone, though often equal amounts.

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A CONTRIBUTION TO THE PHYSIOLOGY AND CHEMISTRY OF THE PARATHYROID GLAND.*

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The present paper records a series of experiments dealing with the production of parathyroid tetany, its mode of origin, and the methods we have employed for its relief. The experiments have extended over a period of two and one-half years, and brief reports have been made on three different occasions during the progress of the work. Because so many publications giving complete reviews of the literature have appeared during the last few months, it is unnecessary to repeat the story of these glands from their discovery by Sandström[1] in 1880 to the present time, and we shall refer only to those researches which have an immediate bearing on our experiments.

Two radically different opinions are held by various investigators regarding the nature and function of the parathyroid glands: First — that they are thyroid rudiments, or, rather, represent the embryonic condition of the thyroid, and may assume the structure and function of the thyroid at times when the latter is called upon to overwork. This view has recently been reiterated by Forsythe,[2] who concludes that, "The parathyroids are glands essentially thyroidal in nature, possessing no peculiar function but engaged in the active secretion of the same substance as the thyroid gland." Second — Other investigators have concluded that the parathyroid glands have a characteristic histological structure, and of function independent physiologically from the thyroid. Although the anatomical relationship is very close, the functional relationship in the mammals is, according to this theory, merely that which exists between all viscera in a

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* Received for publication Jan. 15, 1909.

healthy animal body, but no transformation in structure or vicarious performance of function ever takes place. The parathyroid, accordingly, is a vital organ with a function quite as indispensable as that of the liver or the adrenal. We believe that the second view is the correct one, and it is perhaps suggestive to point out that the first theory has been advanced from the anatomical evidence mainly, while the second is most strongly supported by the physiological and experimental evidence.

Investigators are not agreed either as to the part played by the parathyroid in the body, or upon the precise method of the production of symptoms following complete thyro-parathyroidectomy. Jeandelize[3] has summarized a view which is commonly held, viz.: that removal of the parathyroids causes death from the acute development of tetany, while simple thyroidectomy is followed by chronic cachexia. This generalization has not been accepted in toto by all investigators, and quite recently Thompson and Leighton[4] conclude as a result of some experiments in ligating the gland that this view needs modification. They agree that sudden deprivation of the glands will cause the acute tetany, but hold that a slow destruction results in chronic nutritional disturbances followed by death, without any manifestation of tetany. Macallum[5] has noted that the acute symptoms may be occasionally followed by a stuporous condition, which gradually increases in severity and leads to death without tetany. Our own experiments confirm this observation. Vincent and Jolly[6] report variable and uncertain symptoms following parathyroidectomy and they question the generally described symptom sequence. Their work was done mostly with rabbits, and the impossibility of being certain of the operative procedure vitiates this and many similar observations. In a recent publication one of us (Berkeley)[7] reported the symptoms more marked in young rabbits than in old, but, "whenever we succeeded in finding and removing all the glands, the symptoms developed invariably in a more or less typical fashion.

Occasionally the animal was desperately and typically ill for half a day, but slowly recovered, developing no more symptoms while under observation. After removal of one or two parathyroids, the autopsy always showed that the glands left behind had enlarged — presumably a compensatory hypertrophy. Over seven-eighths of the parathyroid substance must be removed in rabbits in order to produce the characteristic toxemia."

J. Erdheim[8] confirms the belief in the casual relation between parathyroidectomy and the development of tetany. He has worked almost entirely with rats, and in a long series of parathyroidectomies has found in virtually all of them specific symptoms of tetany developing in twenty-four to thirty-six hours following the operation. In three autopsies on patients with tetany following thyroidectomy, he found entire absence of parathyroid glands. The consensus of experimental opinion is therefore in accord with the belief that removal of the parathyroids causes acute tetany, while thyroidectomy is followed by cachexia.

Our own experiments have been made on dogs, and in order to further test the matter we have followed out various operative procedures.* The thyroid and the parathyroid in dogs are in such close anatomical relationship that it is very difficult to remove either entirely without injuring the other. The following diagram shows a common anatomical arrangement of these glands in the dog, an arrangement which we find in fifty per cent of the animals, but it must not be forgotten that in many instances the parathyroid is included in and completely surrounded by thyroid tissue.

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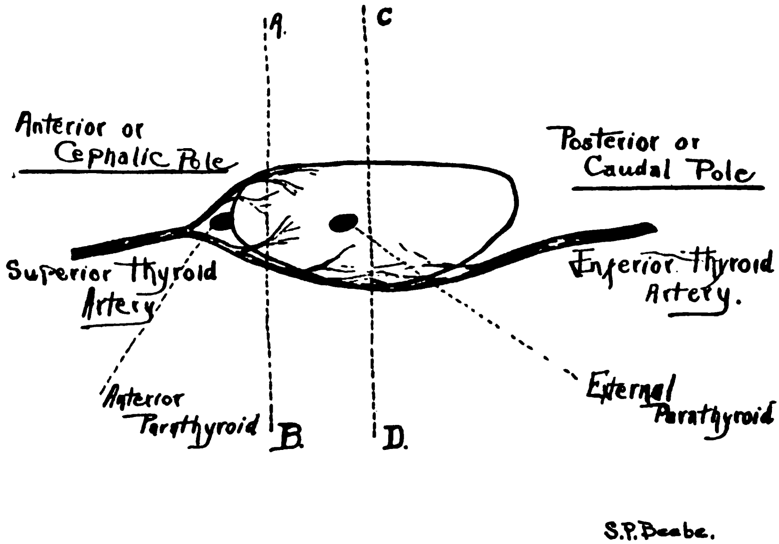
* All the operations on animals have been done under such anesthesia as to warrant the animal being free from pain. If the operation was no more than a skin incision morphine and cocaine have been given, but in more extensive procedures morphine and ether were used.



With this anatomical arrangement in mind, the following operative procedures have been performed with the indicated result in each instance:

1. The entire thyroidparathyroid apparatus has been removed, a complete thyroparathyroidectomy, with the result that the animals have almost invariably developed symptoms of tetany in twelve to forty-eight hours. In two cases, after what was supposed to be the complete operation, there was no development of symptoms and the animals were kept in one instance for six weeks, and in the other for some months without symptoms. Probably an accessory parathyroid not removed was responsible for the nondevelopment of symptoms in these cases, although no such gland was found at the autopsy. Thirty-four dogs were operated upon in this group.

2. A second group of sixteen animals having a complete thyroparathyroidectomy were fed or inoculated with thyroid proteids, in order to determine whether provision for thyroid function would modify the development of symptoms. It is quite probable that such a method of providing the secretion does not answer the physiological need in the same

manner as the natural gland, but even though large amounts of pure thyroid preparations were given, the development of tetany was not delayed, nor were the symptoms modified in any evident fashion. Even the hypodermic injection daily of the extract of two normal dogs' thyroids had no effect in retarding the development of symptoms.

3. A third group of eighteen animals was submitted to operation by resection of a portion of the glands (thyroid and parathyroid combined), in one set the anterior one-half, in the other set the posterior one-half (see diagram). In resecting the anterior one-half of the gland, careful search was always made for the parathyroid on the external surface of the thyroid, and care was exercised to make the dividing line posterior to this glandule (line C–D), so that in a successful removal of the anterior one-half all the parathyroid tissue would be removed and yet an amount of thyroid tissue sufficient to provide for the physiological need of the animal would remain in a normal functional condition. In six out of eight cases in which four parathyroids were seen at the operation and removed with the anterior half, the characteristic symptoms of tetany developed in the usual time. Subsequent removal and section of the thyroid tissue remaining showed it to be in a physiological condition. In those cases in which the posterior half was removed, the dividing line being posterior to the external parathyroid, no symptoms were observed. In fact if the line of section should be made through the line A–B (see diagram) no symptoms were observed, although nine-tenths of the thyroid were removed and in most cases, at least, two of the parathyroids.

4. In a fourth group of nine animals an attempt was made to destroy the parathyroids with the actual cautery, with a minimal amount of injury to the thyroid. In four of these animals we succeeded in finding and cauterizing four parathyroids and in these the operation was followed by the characteristic symptoms of tetany. The cautery caused only a small amount of injury to the thyroid. Microscopic section showed no pathological condition of the latter gland and its

blood supply was not impaired, so that there can be no doubt that it was capable of functioning in these cases, but the symptoms of tetany came on promptly and were quite as characteristic in this group as in the others having a more complete operation. Contrary to Jeandelize, I was unable to see any evidence that tetany came on any more quickly, or that it was more violent in those animals having only parathyroidectomy than in those having a complete operation of thyroparathyroidectomy.

The conclusion to be drawn from these operations is directly opposed to the hypothesis recently reiterated by Forsythe, and supports the belief that the relation between parathyroidectomy and tetany is one of cause and effect. Parathyroidectomy is followed so directly and invariably by tetany that it seems impossible that those investigators who deny the relationship could have been successful in their operative methods. No amount of histological examination or of comparative anatomical study is competent to controvert the experimentally demonstrated fact that parathyroidectomy is followed by tetany while thyroidectomy is not, and the equally positive inference that such a difference necessarily implies a different function on the part of the glands in question.

Origin of the symptoms. — The precise method by which these symptoms are produced is not known. The present ideas in regard to the matter may be summarized in two hypotheses, the toxin hypothesis and the calcium deficiency hypothesis. Some physiologists who admit that tetany follows parathyroidectomy are inclined to believe that the symptoms are caused by a metabolic toxin which is ordinarily destroyed or neutralized by the parathyroid activity. Many of the observed facts may be explained quite satisfactorily by such an hypothesis, but the evidence is not complete enough to formulate a definite theory.

Quite recently the relation of the calcium metabolism to tetany has been the subject of discussion and investigation by Macallum and Voegtlin,[9] and they have formulated what we may call, for purposes of comparison, the calcium deficiency hypothesis. It is more convenient for our purposes to consider the evidence in its favor before presenting facts favoring the idea of a metabolic toxin.

The relation of disturbed calcium metabolism to gastric tetany was suggested by Stöltzner,[10] who believed it to be associated with a "stasis" in the calcium circulation and believed that good results followed phosphorous therapy because of an improvement of the calcium circulation. Cybulski[11] has expressed a somewhat similar belief based on his finding a low retention of calcium in the convulsive stage of tetany and an increased retention during convalescence. His belief that tetany is due to the calcium deficiency in the central nervous system is practically identical with the hypothesis formulated by Macallum. Cybulski was considering tetany of gastric origin, while Macallum's ideas are based practically upon the results of parathyroidectomy, yet there is a striking degree of similarity in the two conditions, and Pineles[12] may be right in insisting that the symptoms in the two cases are due to the same toxin.

Macallum has recently amplified the idea of a disturbed calcium metabolism as the cause of tetany by suggesting that the parathyroid gland in some fashion presides over calcium metabolism, and that surgical removal of the gland is followed by a rapid excretion of calcium amounting to a calcium diabetes, analogous to the dextrose excretion following the total extirpation of the pancreas. Because of this rapid excretion, which is equivalent to the low calcium retention noted by Cybulski, the calcium content of the tissues is reduced to such an extent that tetany results. This rapid excretion has been found in a number of dogs after parathyroidectomy (Macallum), and the evidence of a low calcium retention is found in the fact that a dog dead from tetany had a lower content of calcium in his brain than was found in the brain of a normal dog. Macallum has called attention in this connection to the well known effects obtained by Loeb,[13] Howell,[14] and Sabbatini[15] by depriving excised muscles of calcium and exposing them to solutions of calcium free salts, particularly solutions of pure sodium chloride. Under these circumstances rhythmical contractions occur which may continue for some time, and occasionally leave the muscle in a state of tonic contraction. It has been repeatedly shown that the addition of calcium ions to the solution bathing or perfusing the muscle restores the tissue to a comparatively normal condition and prevents the rhythmical contractions. Sabbatini's observations that intravenous injections of trisodic citrate solutions would cause convulsions and muscular twitching are even more suggestive, because by this means the soluble calcium salts in the body fluids are combined in such a fashion that no calcium ions are available for the physiological reactions in the body (Sabbatini). Probably the most striking argument in favor of Macallum's theory in respect to tetany is his demonstration that the tetany parathyreoprivus may be relieved almost instantly by the intravenous injection of a soluble calcium salt. We have repeated the experiment and find that intravenous injections of a soluble calcium salt will relieve tetany almost instantly; intramuscular injections may be effective after a period of thirty to forty-five minutes, and a similar period is required for the beneficial effect after subcutaneous injection. The effect of the calcium salt when given intravenously to an animal in advanced stages of acute tetany is one of the most striking in the range of physiological experimentation.

We may then summarize the positive evidence in favor of deranged calcium metabolism as the cause of tetany parathyreoprivus as follows:

1. There is a low retention of calcium during the eclamptic period of gastric tetany, a condition probably similar to that of tetany parathyreoprivus (Cybulski, Pineles).

2. Surgical removal of the parathyroid gland is followed by an increased excretion of calcium, and the brain of an animal dead of tetany contains less calcium than is found in the brain of a normal animal (Macallum and Voegtlin).

3. Intravenous administration of a soluble calcium salt promptly relieves the symptoms of tetany parathyreoprivus.
4. The inactivation of the circulating calcium by means of intravenous injections of trisodic citrate is accompanied by muscular twitching and convulsions similar in character to those of tetany, and these may be relieved by the injection of soluble calcium salts in sufficient quantity to restore the equilibrium (Sabbatini).

The very striking demonstration of the complete temporary relief of tetany by intravenous injection of a soluble calcium salt should not lead to the neglect of other possible factors in its production. The evidence in favor of a metabolic toxin being the cause of the symptoms presents many facts of interest, and there are some points which may be considered favorable to either hypothesis. The symptoms in the acute development of tetany point strongly to the action of a poison. The rapid breathing, rapid thumping heart, the nervous activity of the animal, the salivation, the muscular twitching, the evident cerebral disturbance, the final convulsions ending in death with the muscular rigidity passing into rigor are very suggestive of a poison acting on the central nervous system. The muscular twitching is of central origin, for severance of the motor nerve to any group of muscles prevents those muscles from taking part in the tetany (Macallum's observations). The toxin acts in this respect like the poisons of the convulsant series including strychnine, ammonia, the various xanthin substitution products, rather than like physostigmine, which produces its muscular twitching by peripheral action. It may be pointed out that the muscular twitching and rhythmical contractions followed by paralysis, which occur in muscles immersed in calcium free sodium chloride solutions, are examples of peripheral action in muscles severed from the nervous system, and such experiments can hardly be considered competent evidence in support of a theory to explain an action which is essentially central in origin.

It has been noted previously, and our experiments confirm the observation, that young animals show more violent symptoms and show them more promptly following operation than old animals. We have also observed that an animal fed on a meat diet prior to and immediately following the operation shows the symptoms more severely and more promptly than one allowed to fast for two days prior to and following the operation. Feeding on a diet of bread, or milk and bread, is not so unfavorable for the operated animal as a diet containing much meat. We have in many instances observed that the symptoms have promptly ensued in an operated animal one to five hours after a heavy meal of meat. We have occasionally made use of this method to bring on tetany at a favorable time for experimental work. We are not able to state precisely why tetany follows the meat feeding, but we may refer to some facts established by clinical observation which bear on this point. It is well known that a meat diet predisposes toward convulsive seizures in those cases of pregnancy which have disturbances of metabolism characteristic of the preëclamptic state. We frankly admit that the relation between parathyroid tetany, tetany gravidarum and eclampsia is not yet established, but there is a fairly close resemblance in symptoms and there is some suggestive experimental evidence which supports the belief that these conditions are very closely related. In a number of instances, animals (cats and dogs) previously thyroidectomized have had symptoms of tetany during pregnancy or have died with these symptoms at parturition. If the same relation holds for the human species we have collateral evidence in the many metabolic studies which have been made in pregnant patients in the preëclamptic or toxemic state which precedes the occurrence of convulsions. The nitrogen partitions in the urine in such patients are in most cases abnormal, with relatively high ammonia, high rest nitrogen, notable quantities of kreatinin, but with diminished urea and kreatinin excretion. Such findings indicate severe nutritional disturbance, and the possibility of metabolic toxins being responsible for the symptoms in such cases is commonly accepted. The means of relief in acute conditions by the very active stimulation of excretion through skin, bowel, and kidney, through the use of the hot pack, diaphoretics, purgation, the high saline irrigation, vigorous diuresis, and occasionally by bleeding and saline infusion are all based on the belief that the symptoms are caused by an active toxic substance in the circulating blood. A similar method of treatment is of clinical value in the treatment of parathyroid tetany. The fact that under such disturbed conditions of nitrogenous metabolism a meat diet gives rise to metabolic products which may provoke convulsions suggests that the meat diet has more than a passive role in producing the symptoms of parathyroid tetany. Macallum explains the favorable effect of milk as compared with meat by suggesting that the high content of calcium in the milk prevents so rapid a development of symptoms. In this connection it may be remarked that some of our animals on a meat diet were given bone ash and the calcium intake was much larger than is possible on a milk diet, but animals so fed had the same tetanic seizures as those on meat alone. Such an observation is perhaps open to the criticism that the calcium in the milk is much better absorbed than that from the bones. In either case only a small total quantity is absorbed during the time between operation and the onset of symptoms.

In other pathological conditions, notably in diabetic acidosis, there is a very much increased excretion of calcium, in some instances amounting to one hundred per cent more than by a normal person on the same diet, and yet no tetany develops. With so large a reserve supply of calcium as the bones, the demand for this base can be quickly supplied to such an extent as the physiological economy of the body demands; and it seems hardly probable, if this increased calcium excretion can be maintained for the comparatively extended periods that occur in diabetic acidosis without causing symptoms of tetany, that the two days' excretion possible in parathyroidectomized dogs would so exhaust the calcium supply below the limits of safety to the organism as lead to a fatal issue.

Another observation originally made by Macallum which we have repeatedly confirmed is that an animal in severe tetany may be relieved quite promptly by bleeding followed by an intravenous infusion of physiological salt solution. Such relief is only temporary as the tetany begins again in eight to twenty-four hours. Such an heroic method of treatment cannot for obvious reasons be repeatedly applied. If the tetany is due to a low content of calcium in the blood and tissues such a procedure should make matters worse, because the bleeding may remove one-third of the blood with its low calcium content and the subsequent infusion with salt solution dilutes the remaining blood, so that after the operation the tissues are bathed in a fluid having approximately only one-half the calcium content that they previously had and yet the symptoms have abated. Such a method of treatment is, however, well suited to diminish the concentration of a poison in the circulating blood.

Methods of treatment. — The further discussion of the question leads into the third division of our paper, namely, the methods which we have employed for the relief of the convulsions and, after presenting the facts which are of interest in that regard, some of which bear upon the etiology, we shall summarize our own views regarding the metabolic toxin theory.

The demonstration that removal of the parathyroid gland is followed by tetany suggests that this gland elaborates an internal secretion which is of vital importance. It was one of the prime objects of our study to get more information regarding the nature and mode of action of this internal secretion. We cannot exactly imitate the continuous function of a ductless gland by administering at varying intervals extracts of a similar gland taken from an alien species, yet by this method much valuable information has been obtained in respect to internal secretions in other glands. Conflicting opinions are found in the literature regarding the efficiency of extracts of thyroid or parathyroid in arresting symptoms of tetany.

Vincent and Jolly[17] report that they were unable to prevent the onset of symptoms in the complete operation by the use of extracts of thyroid or parathyroid tissue. Moussu[18] made extracts of the horse parathyroid in sterile water and also in glycerine (the former preferred) and found that he was able to arrest surgical tetany in dogs by the subcutaneous and intravenous injections of this gland extract.

The fact that nearly all of the dogs died later in a cachectic condition does not controvert the experimental fact that a temporary complete relief was obtained by these extracts. Macallum[19] reported some beneficial effects to follow the subcutaneous injection of an emulsion of parathyroid gland. Vassalli[20] prepared an extract by a "special method," the details of which he does not give, and found this extract to be active by either oral or subcutaneous administration. He claims to have used it successfully on eclampsia, on infantile tetany, and epilepsy, and thinks it would be good in all kinds of tetany.*

One of us (Berkeley) was able to relieve promptly symptoms of gastric tetany in a man of thirty-nine years (Roosevelt Hospital, service of Dr. W. B. James) by capsules of fresh ox parathyroid ground up with sugar of milk and taken orally. The same preparation gave effective relief in a case of surgical tetany recently reported by Dr. J. J. Putnam.

Our own experiments were undertaken in the belief that the parathyroid furnishes a vital internal secretion, and with the purpose of finding out some further facts regarding the nature and stability of the active agent. Beef parathyroid glands have been used throughout these experiments. An employee of one of the New York abattoirs was instructed by one of us (Berkeley) and soon became quite expert in roughly trimming out a small mass of tissue containing the two upper parathyroid glands. The material was received at the laboratory in this condition, and the glands were carefully freed from adhering fat and connective tissue.

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* Vassalli has evidently believed other laboratory workers incapable of comprehending the fine points in the preparation of his special extract, for he fails to describe his process. Perhaps the allurements of commercial exploitation have prevented a trank presentation of the subject.

The first set of experiments was made to determine whether a soluble principle could be obtained from the gland which would possess the functional activity of the whole gland. The criterion for such a conclusion was the complete relief of acute parathyroid tetany in dogs. The method which we followed in preparing the first extracts was slightly modified later, but has now been followed for a period of several months and has never failed to give active extracts.

The carefully cleaned glands were cut into small pieces with scissors, and the comminuted tissue was then ground to a fine pulp in a large mortar with the help of enough sand to give the whole mass a moist, pasty consistence. The grinding with sand is a slow, laborious process, but the success of the extraction depends in no small degree upon the care with which this portion of the work is done. The crushed glands were next shaken for two hours at room temperature with six to eight volumes of physiological salt solution to which had been added two drops of ten per cent sodium hydroxide solution. The jar was now transferred to the refrigerator and the extraction allowed to continue for eighteen to thirty-six hours. Filtration first through gauze, to remove the fat and coarser particles of tissue, and then through moderately thick paper gave a clear extract which was preserved by the addition of chloroform and kept at low temperature until biologically tested.*

A preliminary experiment proved that an extract so prepared was capable of relieving the symptoms of tetany when given hypodermically. The relief was noted in ten to fifteen minutes after injection of the extract and the animal gradually improved to complete recovery two hours later.

The extract prepared as above described contains nucleoproteids, globulins, and albumins and it seemed to us that we might find that the physiological activity of the gland was carried by one particular proteid. We accordingly divided the proteid in the extract into three portions, the first portion being precipitated by acetic acid, the second by half saturating the filtrate with ammonium sulphate, and the third by complete saturation with ammonium sulphate. Following the common nomenclature we shall speak of these proteids as nucleoproteid, globulin, and albumin.

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* A point of interest in regard to the chemistry of the gland is the fact that we have not found any iodine in any of the parathyroid glands which we have examined. Human, dog, and beef parathyroid have been tested with negative results.

The nucleoproteid is by far the most abundant; the globulin is about one-fifth the nucleoproteid while the albumin is so small in amount that we abandoned any idea of going further with it. The nucleoproteid was precipitated readily by acidifying the extract with acetic acid, and for our purpose it was carefully washed, redissolved in dilute alkali, and reprecipitated twice, with repeated washing to remove any adherent globulin or albumin. It goes into solution readily in a slightly alkaline medium.

The subsequent biological tests permit the following conclusions to be drawn regarding the activity of these proteids:

1. The nucleoproteid of the parathyroid when freshly prepared is equal to the whole gland in relieving the symptoms of acute tetany in dogs.

2. The globulin is of no value in relieving tetany.

3. Boiling the nucleoproteid solution or heating it to 80° C. for one-half hour completely destroys the activity of the nucleoproteid.

4. The nucleoproteid is most active when freshly prepared and rapidly deteriorates when kept in solution or in suspension at refrigerator temperature. Freezing also destroys its activity, although not so rapidly as room temperatures.

5. Tryptic digestion or the action of pepsin and hydrochloric acid for forty-eight hours severely injures but does not completely destroy the activity of the nucleoproteid.

6. The nucleoproteid will relieve tetany if given by mouth, but is much more quickly and certainly effective when given subcutaneously or intraperitoneally.

Details of typical experiments which illustrate these points are given below.

December 7. — Small black mongrel operated, both thyroids and parathyroids being removed. On the days subsequent to the operation sheep's thyroid gland was added to the diet.

Dog continued well until December 10, when he was found at 9.30 A.M. to have very severe convulsions, characteristic of tetany parathyreoprivus. At 9.45 ten cubic centimeters of the solution of parathyroid nucleoproteid containing approximately ten milligrams proteid were given intraperitoneally. In fifteen minutes the dog began to improve and the improvement continued gradually but certainly until at 12.15 he was perfectly well as far as could be detected, and behaved in all respects like a normal dog. The animal continued well and showed no further signs of tetany until December 12, when some indications of tetany were found. These continued to increase in severity until on December 14 at 5 P.M. a small additional dose of two cubic centimeters of parathyroid was given intraperitoneally. Improvement followed immediately and the animal was well the next morning. Slight symptoms appeared again on December 16 and continued to increase in severity until on December 17 the animal was again in a desperate condition. At 12.30, December 17, the globulin from approximately forty glands was given intraperitoneally. This did not cause any improvement and at 3.15 the animal's condition was desperate; accordingly six and one-half cubic centimeters of the parathyroid nucleoproteid solution were given intraperitoneally. In one-half hour there was very marked improvement and an hour later the animal was almost entirely well.

From this point on the dog was given parathyroid nucleoproteid every second day and following each injection the condition markedly improved only to relapse later, but in spite of the injections the dog's general nutritive condition gradually grew worse. He was chloroformed December 24. He was in a markedly cachectic condition, but had been kept free from the convulsive symptoms of tetany by means of the proteid injections.

February 18. — Complete thyroparathyroidectomy. Animal given hypodermatic injections of sheep thyroid proteid twice each day (five cubic centimeters of a two per cent solution). Animal was kept on a meat diet and remained well until February 20, when marked twitching was noted. This increased, but no convulsions were noted. February 21, while the animal was being closely observed, he gave a sudden cry and fell over in the cage unconscious, respiration stopped, and the heart beat only six times per minute. It was evident that a fatal termination was at hand, but artificial respiration was begun after three minutes delay, and at the same time an intravenous injection of parathyroid nucleoproteid was given. In five minutes spontaneous respiration began again, and in ten minutes consciousness returned with slight twitching of the muscles. The latter gradually decreased, and in one-half hour from the onset of the attack, the animal was running about the laboratory in a quite normal fashion. One-half hour later the symptoms of tetany again appeared, and it was necessary to repeat the injections of the parathyroid nucleoproteid. The symptoms were promptly relieved. From this time on the animal was kept free of tetany by parathyroid administration, but his general nutritive condition suffered so much that he was chloroformed on March 5.

March 8. — Small woolly dog; operation was the removal of the anterior two-thirds of the thyroid on both sides, taking at the same time both parathyroids. The cut surface of the posterior third was cauterized. A sufficient amount of normal thyroid tissue was thus left in situ to answer the physiological need of the animal. Symptoms of tetany began March 10, and increased gradually to such a point that on March 11 an injection of five cubic centimeters parathyroid nucleoproteid was given. The symptoms were promptly relieved. March 12 at 1 P.M. marked symptoms having again appeared, five cubic centimeters of the parathyroid nucleoproteid solution, which had previously been heated to boiling, were given. (The proteid is not coagulated if heated in alkaline solution.) This injection had no perceptible effect upon the symptoms, and accordingly after four hours interval, during which time they became progressively more pronounced, five cubic centimeters of the unheated nucleoproteid solution were given. Within fifteen minutes the animal showed improvement, and in two hours the animal was free from tetany. Two days later the symptoms recurred. The boiled solution again failed to relieve, while the unboiled caused prompt disappearance of symptoms. On March 18 the animal was chloroformed. The remaining third of the thyroid was found normal both in the gross and after microscopic examination.

The effect of the parathyroid nucleoproteid in relieving tetany has been tried upon thirty-two animals having typical symptoms of tetany. The results have been positive in ninety-five per cent of the trials, providing a comparatively fresh preparation was used. Eight animals have been given the boiled proteid without showing any relief. In nine the products of peptic or tryptic digestion have been given with beneficial effect, but not to the same extent as with the undigested proteid. Complete relief may be obtained with these digested proteids, but a very much larger dose is required. The globulin has been used upon nine animals, always with a negative result. It does not seem necessary to put on record the details of each of these separate experiments.

The clinical value of the nucleoproteid has been tried in two cases of parathyroid tetany in the human subject.

One, reported by Halstead (in the American Journal of the Medical Sciences, July, 1907), had been in a miserable condition for some months, and had received such treatment as was then available. The injections of parathyroid nucleoproteid were continued for some time and resulted in a very gratifying improvement in the patient, so that she was no longer the helpless invalid she had been since her operation.

The second, reported by Poole (Annals of Surgery, October, 1907), was a seamstress who had had two thyroid operations. Following the second typical tetany developed within four days and continued for thirteen months in spite of the usual lines of medical treatment, in addition to Vassali's serum and implantation of the parathyroid gland. Improvement finally occurred and "was coincident with the repeated administration of Beebe's nucleoproteid by hypodermic injection in large doses."

Experiments with calcium, strontium, and barium salts. — We have confirmed Macallum's observations that intravenous injections of calcium salts give complete temporary relief from the symptoms of tetany and we were interested to know in how great a degree this might be a specific effect. Calcium belongs in a natural group of metals, containing also magnesium, strontium, and barium, which have many similar chemical properties. Macallum found that magnesium salt[5] would also relieve the tetany but that the depressant action of the magnesium was so marked that it was a dangerous salt to use. Strontium salts are, however, free from the severe depressing action of the magnesium compounds and are perhaps even less toxic than the calcium salts. We therefore made a series of experiments to determine the effect of intravenous injections of strontium salts upon tetany. Solutions of strontium chloride containing two, five, and ten grams per one hundred cubic centimeters were used for this purpose. Details of two typical experiments are given below. Our results show that fairly large doses (ten cubic centimeters of a ten per cent solution of strontium chloride to a ten-kilogram dog) may be given without noticeable toxic effect. Furthermore, the symptoms of tetany are relieved with nearly the same degree of promptness and completeness that follows the injection of calcium salts. The effect of strontium salts has been tried upon ten animals in tetany, and it is scarcely possible to decide whether calcium salts give better results. The effects of the calcium appear a little earlier than those of the strontium, but they are not more complete or lasting.

Experiment Z. August 1.—Young male dog, seven kilos, complete thyroidectomy. Glands rather large, weighing two grams each.

No symptoms were noticeable until 6 P.M. the next day when the onset of tetany was plainly evident. At this time five cubic centimeters of a ten per cent solution of calcium lactate were given into the muscles of the back. The symptoms gradually subsided.

On August 3 symptoms began at 10.30 A.M. and increased gradually in severity until 5.30 P.M., when they were very pronounced. At this time five cubic centimeters of a five per cent solution of strontium chloride were given intravenously. All the symptoms were immediately controlled. The animal ate and drank and appeared to be in a normal condition.

On August 4 slight twitching noticed at 10 A.M., and this gradually increased in severity until 11.50, when the condition of tetany was very pronounced. Five cubic centimeters of a ten per cent solution of strontium chloride given into the muscles of the back gave a very prompt relief.

August 5.—Slight twitching noticed at 8 A.M., symptoms gradually increased to great severity at 10.30 A.M. Five cubic centimeters of strontium chloride at 10.30 were followed by very prompt relief.

Notwithstanding that the symptoms could be kept under control by the injections of strontium chloride, the animal's nutritive condition became progressively worse, so that on August 6 the animal was chloroformed.

Barium salts likewise will relieve tetany, but the barium compounds are so toxic that only a small dose can be given without fatally injuring the animal. Nevertheless, it is possible to give an intramuscular injection of barium chloride and get fairly prompt relief from symptoms of tetany. The heart and gastrointestinal tract are powerfully stimulated, however, and the dose must be carefully regulated as animals differ somewhat in their susceptibility. All four of the metals in this group are effective in relieving the symptoms of tetany. Strontium and calcium salts are both comparatively harmless agents and may be given intravenously with perfect safety. Magnesium at one end of the scale is a powerful depressant, while barium at the other end is a powerful stimulant and they are both dangerous to life when given in a dose sufficient to control the symptoms of tetany. Calcium and strontium occupy comparatively neutral positions.

The relief of symptoms by injections of barium salts is not an easy matter to demonstrate for the reason that an efficient therapeutic dose and a fatal dose are so near together. In several instances we have been successful in relieving the symptoms only to find that fatal barium poisoning ensued. The relief of tetany by barium salts is therefore of theoretical interest only for the reason stated. The effects are not as prompt as with calcium and strontium salts and the dose which different animals may safely take varies somewhat. It need hardly be said that *barium salts should never be given as a therapeutic measure for the relief of tetany.*

August 5.—Young fox terrier, weight, 6.5 kilograms; total thyroidectomy.

August 7, 4 P.M., well developed tetany. Canula placed in external jugular vein under eucain anesthesia. Normal saline was allowed to flow in slowly while the hypodermic syringe containing the barium salt was slowly emptied into the rubber leading to the canula.

One cubic centimeter of a five per cent solution of barium chloride was thus introduced. There was no appreciable effect in five minutes.

One cubic centimeter additional was then introduced and the animal at once became quiet. The animal remained free from tetany until August 8 at 5.30 P.M., when similar treatment (1.5 cubic centimeters of five per cent solution of barium chloride given in the manner described above) again caused prompt relief. Following this experiment the animal was used for a different phase of the work and was not again treated with the barium salts.

The fact that tetany can be relieved by these various means suggests that the symptoms do not arise because the concentration of calcium is reduced to so low a point that the tissues are bathed in a correspondingly large quantity of potassium and sodium salts. We know that strontium may, to a certain extent, replace calcium, that it is deposited in the bones, and it may be effective because of its close chemical and physiological relationship to calcium.

However, it seemed to us probable that the calcium and strontium both acted in some measure as sedative or depressant upon some portion of the central nervous system which had been unduly stimulated by a toxic substance arising in the course of intermediary metabolism. The depressant action of calcium is well expressed by Sollman,[21] who says, "The action of calcium is directly depressant to all tissues and functions. When injected intravenously it paralyzes the central nervous system, lowers the blood pressure, arrests diuresis and peristalsis." This depressant action is not always manifest, as for instance its action in causing immediate relief and apparently complete restoration to a normal condition from the profound depression caused by magnesium salts. Meltzers'[22] experiment is quite as striking as Macallum's, though it apparently shows a paradoxical action of the calcium. Possibly the explanation in each instance lies in a higher degree of affinity which certain cells of the central nervous system may have for the calcium, the calcium thereby displacing not only the magnesium but also the metabolic toxins.

It occurred to us that it would be of value in this connection to try the effect of calcium and strontium salts upon the toxic effect caused by a number of substances, as strychnine, caffeine, ammonia, and xanthin which, presumably, act upon the same mechanism as the hypothetical metabolic toxin of tetany. Conflicting reports are found in the literature in respect to the effect of calcium upon strychnine poisoning Our own experiments did not show much antagonistic action for the calcium. If less than fatal doses of strychnine were given intravenously we obtained by calcium injections a more rapid diminution of the reflex excitability, but we have not been able to save an animal from a certainly fatal dose of strychnine given intravenously. Likewise the experiments with caffeine were not conclusive. A comparatively large dose of caffeine is required to produce convulsive symptoms in dogs. In those experiments in which sufficiently large doses of caffeine to produce severe convulsions have been given intravenously, a fatal result has supervened before calcium salts could be injected. If only the milder symptoms were produced the calcium or strontium salts afforded fairly prompt relief. In two experiments a large preliminary injection of calcium lactate, in one instance, and of strontium chloride in another was followed by intravenous injections of caffeine citrate. In these two animals fairly marked stimulation was obtained, but not the fatal convulsions which had been produced by a dose of similar size in another animal.

The toxic effects of ammonia and xanthin when given by intravenous injection are very promptly relieved by the calcium and strontium salts. The relief is not quite as prompt as is the relief of the symptoms of tetany, but in from one to three minutes the very marked muscular twitching is entirely relieved and the animal is normal in its behavior. It is possible to regulate an intravenous injection of ammonium salt, either the carbonate or the acetate, so that the animal is continuously in a mild state of stimulation, but at no time has dangerous symptoms. As soon as one stops the injection the excessive stimulation ceases and the animal gradually resumes a comparatively normal condition. The ammonium salt is apparently used in urea synthesis with remarkable speed. If one oversteps the safe minimum, however, very severe toxic convulsions result and a fatal termination promptly ensues. With nonfatal doses fairly prompt recovery follows the cessation of ammonia administration. Fatal doses cause death as promptly. If one gives the intravenous injection of strontium or calcium chloride immediately after the injection of a minimum fatal dose of an ammonium salt, the symptoms are at once arrested and the animal quickly regains a normal condition. Preliminary injections of strontium chloride protect the animal from a subsequent injection of a large dose of ammonia without causing symptoms.

The injection of xanthin produces symptoms somewhat similar to those of ammonia, but the recovery from nonfatal doses of xanthin is slow. The alleviation of the symptoms of xanthin poisoning by the strontium and calcium salts is undoubted, but the experiment is not quite as striking as that with ammonia for the reason that three to five minutes are required to completely relieve the animal.

Typical experiments are recorded below:

August 10. — Mongrel, weight, eight kilograms, given 1.5 cubic centimeters of a two per cent morphine solution. Needle placed in external jugular vein through which injection could be made. Solution of ammonia carbonate containing 1.6 per cent ammonia used for injection. Injection began at 10.36 and continued to 10.58, during which time animal received fifty-four cubic centimeters of ammonium carbonate solution. Symptoms began promptly and continued to increase gradually in severity until the appearance of convulsions at 10.58. At that time eight cubic centimeters of a ten per cent solution of strontium chloride were given intravenously. The convulsions were relieved at once and the animal was normal at 11.02. Injections of ammonia begun again at 11.13. Between 11.13 and 11.18, forty-one cubic centimeters of the ammonium solution were given without the further production of symptoms.

August 15. — Four-kilogram dog, one-fourth of a cubic centimeter of two per cent morphine solution. Canula placed in femoral vein using eucaine as an anesthetic. A solution of xanthin containing 3.6 milligrams per cubic centimeter used for injection.

Injection began at 2.33, continued slowly until 3.06, during which time fifty-five cubic centimeters of the solution containing one hundred and ninety-eight milligrams of xanthin had been given. At that time the animal was in convulsions, the jaws snapping and the muscles all over the body twitching vigorously.

Between 3.06 and 3.08, eight cubic centimeters of a ten per cent solution of strontium chloride were given intravenously, and at 3.10 the convulsive movements had entirely ceased. The limbs were slightly rigid for three minutes longer and then the animal was in a perfectly normal condition. Control experiments showed that without treatment the muscular and toxic symptoms produced by a dose one-third less than the one given above would continue for thirty to fifty minutes, after which time the animal slowly recovered.

These experiments are not cited as proof that the symptoms of tetany are caused by ammonia and xanthin poisoning. Much more evidence must be produced before we can consider such a conclusion. We have demonstrated, however, that these two simple substances produce symptoms which are closely related to those of tetany, known to be of central origin, and we have shown that they may be promptly relieved by the intravenous injection of calcium or strontium salts in the same manner as the symptoms of tetany.

The metabolism experiments recently reported by Underhill[28] and confirmed by our own findings show there is a much increased excretion of ammonia following the complete thyroidectomy.

It seems to us, therefore, that ammonia may take some part in the production of the symptoms of tetany. The symptoms of ammonia and xanthin poisoning are readily produced in an animal having a normal quantity of calcium in the blood, and are relieved by the further addition of calcium or strontium indicating that the protection may involve a reaction in which the mass action of the extra calcium or strontium plays a part. In other words we believe that the essential fact in the production of symptoms following complete thyroparathyroidectomy is the deranged metabolism giving rise to some active poison, and not the abnormal excretion of calcium which we regard as an accompanying phenomena, perhaps due in part to the starvation cachexia which ensues in most cases if the animal is tided over the acute condition.

We may summarize, then, the arguments in favor of regarding the symptoms as due to a metabolic poison:

I. The symptoms have a central origin.

II. Symptoms are shown best in young animals and are more severe if the animal has been kept on a meat diet.

III. The symptoms have close relation to certain clinical conditions which are accompanied by severe nutritional disturbances.

IV. Gastric tetany is accompanied by severe metabolic disturbance, it has similar symptoms and is promptly relieved by intravenous calcium injections and by parathyroid nucleoproteid.

V. Bleeding, followed by intravenous infusion, relieves the animal, a procedure well suited to free the body from a circulating poison.

VI. Symptoms are promptly relieved by the injection of fresh parathyroid nucleoproteid.

VII. Injection of known simple poisons such as ammonia and xanthin produce symptoms which can be promptly relieved by injection of calcium or strontium salts, similar to the relief obtained by the same means in tetany.

VIII. Parathyroid tetany has a deranged metabolism accompanied by a large increase in the excretion of ammonia.

We do not wish to go on record as of the opinion that the various factors in the etiology of tetany are settled. The experiments here reported are a portion of an investigation which is still in progress and with additional experience our opinions may change. From the information at our disposal at present we are inclined to believe that the parathyroids are chiefly concerned in furnishing enzymes which are of prime importance in the intermediary metabolism of nitrogen.

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A TRANSMISSIBLE CANCER OF THE RAT CONSIDERED FROM THE STANDPOINT OF IMMUNITY.*

FREDERICK P. GAY.

The occurrence in mice and rats of conditions of apparent immunity or resistance to transmissible tumors has attracted due attention from the beginning of the modern experimental investigation of cancer. The lack of definiteness of many results bearing on this aspect of cancer study is owing not so much to lack of consideration as to the inherent difficulty of the problem. The difficulty is due to the multiplicity of known or unknown conditions that modify any given experiment.

This study makes no claim to completeness in any given part, the confused and often apparently irreconcilable results of other observers, having made a somewhat skirmishing method of attack for the purpose of orientation, seem most serviceable. The reply of many of the experiments is negative or doubtful, and some of them undertaken to answer apparently the most logical inquiry are so problematical in result that they will not even be mentioned. As a result of the work has come the knowledge of certain conditions that are controllable, as well as distinctly promising data that merit more careful study.

In order to simplify experimental conditions as far as possible most of my work has been done with a single strain of tumor, a tumor of the rat already described by Flexner and Jobling[1] to whom I am indebted for a transplant. This tumor originated in the seminal vesicle of a white rat and, as found, was described by Flexner and Jobling as a mixed cell sarcoma; they have since noted its transformation after several generations into a "malignant adenoma" or carcinoma. Transformations have been described in mouse tumors by Apolant, Ehrlich and Haaland,[2] and by Loeb[3] (Carcinoma

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* Work performed under a grant from the Cancer Commission of Harvard University. Received for publication Jan. 16, 1909.

transformed into Sarcoma) and in a rat tumor by Lewin[4] (Carcinoma to Sarcoma in a few generations). The Flexner-Jobling tumor as it came into my hands may be described as an adeno-carcinoma containing a considerable amount of connective tissue; it has not changed appreciably in histological structure during the eight generations through which I have cultivated it.

Reference will be made to a few incidental experiments that have been made with the Ehrlich and the Jensen tumors of mice. The rat tumor has been most serviceable on account of the larger size of the host (which permits the collection of an appreciable amount of blood), the rapidity of the growth of the tumor, and the frequently and relatively rapid occurrence of metastases.

The rats have been inoculated by means of a trochar needle inserted subcutaneously along the abdominal surface and pushed cephalad as far as the axilla, at which point the "load" of the needle is expelled. It has been emphasized by Bashford, Murray and Cramer,[5] and by Bridré[6] that inoculation at this point results in the largest percentage of takes, probably owing to the vascularity of the region. The tumors used for transplantation have purposely been of considerable size, say from one and one-half to two centimeters in diameter; it has been noted by Jensen[7] that small tumors are less effective for implantation than older ones, probably owing to the fact that actively mitotic cells are less resistant. Tumors of this size correspond to a growth of from four to six weeks and contain a large soft center of broken down detritus surrounded by a more or less considerable shell of actively-growing tissue. It has further been pointed out by Clowes and Baeslack[8] that such broken down tumors are characteristically more "virulent," which fact they correlate with an increase of calcium and absence of potassium. Tumors that have grown to this stage tend to become infected through skin abrasions and become sloughing and foul smelling, although the living periphery apparently continues to grow with undiminished vigor; such infected tumors have been avoided in transplantation. After killing the rat with chloroform, the tumor to be transplanted is removed with aseptic precautions and the growing shell of tumor removed with sterile scissors and cut into small pieces. The importance on the result of the amount of tumor used for inoculation has been recently emphasized by Bridré, and by Gierke,[24] who consider that many apparent discrepancies between results obtained by different observers may be entirely attributable to this factor. Without employing an emulsion of cells, which from the observation of others seems less suitable for the transplanting, or without weighing the small bit of tissue injected, which seems a well nigh impossible refinement, there has appeared to be no way of obtaining more than approximate uniformity of dosage. An amount of living tissue equal to approximately half a match head in volume has been used for each animal. The variations in amount incident to this method are not, in my judgment, sufficient to render my own results other than comparable and are certainly not so marked as the variations between different observers.

In all later experiments young rats weighing from fifty to seventy-five grams have been employed as they are admittedly more susceptible than adult animals (Bashford, Murray and Cramer, Lewin and others). The source of supply, or race of white rats used, has seemed to be of very distinct importance in reference to the percentage of successful inoculations. Such racial differences of susceptibility have been particularly emphasized in the case of mouse tumors by Haaland,[9] who found that a simple change of locality was often sufficient to render individual races either more or less susceptible. Lewin has, however, noted no such differences in racial susceptibility to his rat tumor. Jobling (personal communication) has found no differences in the percentage of "takes" of his tumor in rats from different dealers. With such definite information as regards rat tumors I was not prepared to meet with the marked variations in racial susceptibility which have been evident in my hands. Many experiments have been rendered inconclusive through a failure to appreciate this factor from the beginning. During the last few months it has unfortunately been impossible to obtain animals of the most susceptible strain. In the following table is summarized my findings in this respect. The animals included are only such as have received no treatment designed to influence the growth of the tumor. Not all the strains of rats were given any individual tumor, as would be desirable if an experiment for determining this racial variation alone had been planned. It is obvious that the "virulence" of the tumor may vary from generation to generation, and that no given tumor is strictly comparable with any other. In order to allow for this variable factor the number of different tumors used in implanting each given race of rats is included in the following summary:

TABLE I.

Percentage of positive inoculations in rats obtained from different sources with the Flexner-Jobling tumor.

Source.	No. of Different Tumors.	Total of Rats.	Positive.	Negative.
A. Adult rats (100 grams +):				
C.	7	25	25	0
P.	2	8	1	7
W.	1	2	0	2
B. Young rats (50–75 grams):				
P.	2	20	2	18
W.	4	8	2	6
H.	1	3	0	3
L.	8	140	70	70

It will be seen that whereas not a single rat from source C has failed to take a tumor that the next best source, L, took in only fifty per cent of the cases and in the animals of source P only three out of twenty-eight gave positive transplants.

Visible metastases, as has been noted by Flexner and Jobling, occur regularly, following successful transplants of the tumor. These authors by means of transplants into several regions obtained metastases in the lungs, peribronchial nodes, and kidneys. Using the subcutaneous axillary transplantation, I have obtained metastases in the lungs and peribronchial lymph nodes usually, and in the adjacent axillary lymph nodes only rarely. The metastases of the lungs may be so extensive as to invade and replace an entire organ. The time of their occurrence after inoculation would seem to be of considerable importance, as will be found in a later consideration of re-implantation of tumor-bearing animals at successive periods.

In considering the time after implantation at which metastases occur, there is evidence that rats of the most susceptible strain produce metastases earlier than do those of a more refractory strain. The fact that different individual tumors and also successive generations of the original tumor were employed with presumable differences in infectivity, render this comparison of the metastasis formation not strictly exact. It was noted that during six generations of the tumors and as a result of implantations from seven different tumors, one hundred per cent (*i.e.*, twenty-five) of the adult rats from dealer "C" showed tumors. The metastasis formation in such of these animals as were used as controls and not subjected to influences that might affect tumor growth, was as follows:

In three animals killed on the twenty-fifth, thirty-fourth, and thirty-seventh day after implantation no metastases were found. In eleven animals killed from the thirty-seventh day (3) to the sixty-eighth day metastases were found in all.

The young rats from source "L" implanted from eight different tumors took the tumor in fifty per cent (seventy out of one hundred and forty).

In this less susceptible strain, metastasis formation would seem to occur later and with greater irregularity than in the "C" rats, thus:

Of six tumor rats killed on the fifty-eighth to sixtieth day metastases were present in four, and of twelve animals killed from the ninetieth to the one hundred and fifty-sixth day, metastases were present in six only.

This summary, then, would seem to show that in rats moderately susceptible to the tumor, metastasis formation is more delayed even in those animals in which the tumor has "taken."

The metastatic nodules in the lungs resemble the original growth histologically, and I have tried to determine whether such metastases would prove equally or perhaps more virulent than the original tumor. A rapid transformation of the tumor in a few generations back to its original sarcomatous form was also a possibility. In the following generations rats from the same highly susceptible source (C) were used throughout:

I. — Rat F. B. 5, bearing a tumor that had been growing five weeks was chloroformed and the tumor used to inoculate.

II. — Rat F. C. 4, which was killed on the fifty-fifth day and showed many metastases in lungs and about heart.

III. — Rat F. D. 15 was implanted with lung metastasis from F. C. 4 on right side and with bits of original tumor from the same animal on the left side. The tumors grew equally well on both sides. Animal killed on thirty-seventh day showed many metastases in both lungs.

IV. — Rat F. E. 9 was given metastasis from F. D. 15 subcutaneously, and Rats F. E. 2 and F. E. 7 (among others) were given the original non-metastatic tumor from F. D. 15. On the thirty-seventh day all three were killed. The tumors in F. E. 2 and F. E. 7 measured 2.6 x 2.2 and 2.2 x 1.9 centimeters, respectively, and in F. E. 9, 4.25 x 3.5. F. E. 2 showed two small metastases on lungs. F. E. 7, no metastases. In F. E. 9 both lungs were filled with metastatic nodules. The original tumors F. E. 2 and F. E. 7 had both suffered necrosis through the skin. The tumor in F. E. 9 although larger had not broken through, and was firmer in the center. Histologically F. E. 9 differed markedly from the other two, being much more cellular and containing less connective tissue.

V. — Rat F. F. 46 was given lung metastasis from F. E. 9. The tumor grew slowly and remained firm. On the sixty-first day it measured 4 x 3 centimeters, and the animal was chloroformed. The tumor had a firm necrotic center. The pleura of both sides and both lungs with the exception of the right lower lobe were entirely replaced by metastases. This tumor also was found histologically to contain more epithelial cells and less connective tissue than the ordinary tumor.

The impossibility of obtaining any more rats from this susceptible source for several months has unfortunately enforced the abandonment of experiments along this line. The metastatic tumor showed no greater infectivity in normally resistant rats than the original tumor.

There was no histological variation of cell picture other than the relative variations noted found in the original tumors nor in the metastases during these five generations. There would appear to be a distinct increase of virulence in the metastatic tumor as noted under IV. (*i.e.*, second generation of successive implantation from metastases compared with corresponding generation from original tumor). The tendency of the "metastatic" tumor to remain firm and uninfected, irrespective of tumor or size, seemed characteristic (IV. and V.), and differs from the picture of the non-metastatic tumor which is usually sloughing at this time. The relative increase of the epithelial elements in the last generations has already been mentioned.

The fact that transplantations from metastases "take" at least equally as well as transplants from the original tumors has so far as I am aware not been definitely stated by any one save Murray.[25] The entire question merits more attentive consideration.

In his first report on mouse tumors Jensen attributed the failure of fifty per cent of mice to take his tumor to a natural immunity. Clowes and Baeslack [10] noted that mice that have spontaneously recovered from the Jensen tumor are immune to subsequent implantation with the same tumor. That such an immunity is at least relatively perfect is now generally admitted. In the experience of Bashford, Murray and Cramer the immunity following the resorption of the first tumor is absolute for the same tumor and partial for other tumors. Bridré regards this immunity as only relative, for on second inoculation some of his animals took; he found, however, that none that had resisted two inoculations took a third. Michaelis [11] also regards such immunity as distinct but relative. Sticker,[12] who worked with a sarcoma in dogs, found that the sixteen per cent that had recovered from a first implantation were absolutely immune to a second. The question of this condition of increased resistance is complicated somewhat by the normal resistance to first inoculation. To what extent can the failure of the first tumor to grow completely without retrogression be attributed to a normal immunity and to what extent to an active acquired immunity following the implantation of the tumor? Can we assert that no tumor which has grown beyond such and such dimensions will never retrograde? Of course when a large tumor has grown and then disappeared it may be assumed to have been resorbed, although even under these conditions Gierke[13] has described tumors which, although apparently entirely resorbed, began to grow again on the inoculation of a second tumor. It is further evident that without control animals any time limit as to when a tumor may be judged as positive must be arbitrary.

In order to increase to the maximum the resistance in certain animals for purposes to be later detailed I have reimplanted tumors in rats that failed to take the first tumor, at two successive periods (from ten days to a month apart in different lots). Of sixteen such animals only one ever developed a tumor and that one only on the third implantation. It is of interest to note that the single tumor that took on the third trial was a metastasis from "metastatic" tumor F. F. 46 (see page 180).

Ehrlich[14] found that if spontaneous hemorrhagic tumors, which grow in only a small percentage of animals, are implanted, instead of tumors of high virulence, that the animals so treated are immune to virulent tumors.* And they are immune not only to virulent tumors of a cellular type similar to the one used for immunization but also to tumors composed of other types of cells. In other words, the immunity acquired is not absolutely specific, and for this reason has been referred to by Ehrlich as a "pan-immunity." As is usual in all matters pertaining to this complex problem other experimenters have obtained only relatively similar or even directly opposite results. Bridré, Haaland, and Michaelis find that treatment with one tumor will not protect against a tumor of a different type. Bridré, however, obtained an almost absolute immunity against mouse tumors by several subcutaneous injections of macerated tumor. The apparently divergent results of various observers are wisely attributed by this writer to differences in individual technic; Ehrlich, for example, used much more tumor material for preventive implantation than did Bridré. It has been clearly shown by Michaelis and by Clowes[15] that immunity can be produced only by the living cancer cells and not by those killed with heat or chemicals, nor yet by the nucleoproteids derived from them. We may conclude, then, that an undoubted increase of resistance to tumor implantation may be produced in rats and mice by the previous implantation, with or without temporary growth, of the same type of tumor, and perhaps, under certain conditions, of other tumors.

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* It has recently been shown by Murray that these tumors will transplant if smaller amounts of tumor are used.

The next advance of interest was the determination that this condition of more or less perfect immunity may also be produced by the injection of certain normal tissues from animals of the same species. Bashford, Murray and Cramer found that mice previously treated with the whole blood (or simply with the red blood cells without serum) of normal mice were protected from mouse tumors in a large percentage of cases. These results have been confirmed by Bridré for mouse tumors and by Lewin for rat tumors. Schone[16] obtained similar protection by injecting mouse embryos. Bridré found an almost perfect protection following the injection of mouse spleen and a somewhat less one following mouse liver. The pan-immunity of Ehrlich which followed the implanting of virulent hemorrhagic tumors and the failure of these tumors to take in many instances may be attributed, according to Gierke, to an immunity caused by the blood in the tumor.

The exact nature of this immunity following resorption of a cancer or implantation with cancer or normal tissue has been attentively considered. Bridré has described it as an "iso-immunity to mouse tissue." The original assertion of Gaylord, Clowes and Baeslack[17] that the serum of recovered mice will cure cancer in other mice, or, when mixed with cancer, prevent its growth, has received no confirmation. The experiments of Crile and Beebe[18] on transfusion of blood from dogs recovered from lymphosarcoma into dogs suffering from the same tumor, followed in certain instances by an apparent cure, do not seem to me to indicate necessarily the presence of immune principles in the blood of the donors. Controls transfused with blood from normal dogs seemed to give almost equally good results and the fact that in most of the cured cases more blood was transfused than had been removed from the recipient would seem to be a factor of even more importance than they have given it. There are, moreover, several observations which would seem to prove that the blood of such refractory animals does not contain actual antibodies for cancer cells. Michaelis[19] studied the serum of actively immunized mice and found that it did not agglutinate nor destroy cancer cells. He found, moreover, that a combination of this serum with cancer extract effected no alexin (complement) fixation. Sticker found that the blood of dogs recovered from his sarcoma did not agglutinate sarcoma cells. Flexner and Jobling found that the serum of recovered or repeatedly implanted rats had no power to protect normal rats from their tumor.

It is naturally a matter of extreme importance to determine whether this acquired or intensified immunity is similar to the usual form of immunity against bacteria, that is, whether it is characterized by the presence in the serum of immune substances antagonistic to cancer cells. The most delicate means of detecting such antibodies is the Bordet-Gengou "reaction of fixation" method. The reaction depends essentially on the fact that the complex, immune body plus antigen has the power to absorb the alexin (complement) of fresh serum, the absorption or non-absorption being proved by the absence or presence of hemolysis in subsequently added, sensitized red blood cells. In the instance in question the fixation of the alexin in fresh serum by a combination of cancer extract and the serum of a refractory mouse or rat would prove the presence of antibodies to cancer in that serum. I have performed a number of experiments of this sort fully controlled in the manner indicated by the following experiment:

EXPERIMENT I.

A refractory Rat F. E. 12 (having received two implantations of the tumor without result, the last time sixteen days previously) and a normal rat were bled from the carotid on May 18. The respective sera were separated from their clots on the following day and heated to 56° C. for one-half hour (to destroy alexin). .1 gram of rat cancer that had been dried at 37° C. was shaken up in three cubic centimeters of NaCl solution (.85 per cent) and after repeated filtration used as indicated in preparing the following tubes:

Tube	1.	Serum of refractory rat, 56°,	.3	cc.*	+ cancer extract	.05	cc.†
“	2.	“ “ “ “ “	.2	“	+ “ “	.05	“
“	3.	“ “ “ “ “	.1	“	+ “ “	.05	“
“	4.	“ “ “ “ “	.3	“	+ NaCl sol.	.5	“
“	5.	“ “ “ “ “	.2	“	+ “ “	.5	“
“	6.	“ “ “ “ “	.1	“	+ “ “	.5	“
“	7.	“ “ normal “ “	.3	“	+ cancer extract	.05	“
“	8.	“ “ “ “ “	.2	“	+ “ “	.05	“
“	9.	“ “ “ “ “	.1	“	+ “ “	.05	“
“	10.	“ “ “ “ “	.3	“	+ NaCl sol.	.5	“
“	11.	“ “ “ “ “	.2	“	+ “ “	.5	“
“	12.	“ “ “ “ “	.1	“	+ “ “	.5	“
“	13.	NaCl.	1	“	+ cancer extract	.05	“

To each tube was then added .1 of a cubic centimeter of fresh rabbit serum (eighteen hours old) and after contact for five hours at room temperature, .5 of a cubic centimeter of a five per cent suspension of sensitized guinea-pig corpuscles ‡ was added to each tube. Hemolysis (two hours at 37° C.) was complete in all tubes but 1, 2, and 7 in which latter it was shortly after complete.

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* Tubes in this column are made with salt solution to equal in volume one cubic centimeter.

† Tubes in this column are made to equal in volume .5 of a cubic centimeter.

‡ This is washed guinea-pig's blood in five per cent suspension that had been mixed for several hours with the inactivated (56°) serum of a rabbit that had received four injections of guinea-pig blood (hemolytic serum). Proportions fifteen cubic centimeters blood suspension to three cubic centimeters of serum.

There is then no distinct evidence that the blood of this refractory rat contained any antibodies for cancer cells.

In some experiments the extract obtained by maceration from a fresh tumor was also tried in place of the extract from dried tumor. The factors involved (antigen (cancer extract) and the suspected antiserum) were varied in amount; another reactivating serum (alexin) was tried; and other hemolytic combinations employed (viz., rabbit blood plus antiserum from the guinea-pig, and sheep blood with antiserum from the rabbit).

In this manner the blood serum of six different rats that had been rendered highly refractory to the tumor and also of three animals of a normally refractory strain were tested for antibodies and none were found. The "pooled" serum of several mice that were rendered refractory to the Ehrlich tumor (Stamm II.) was also tested with this tumor with a negative result.

There is no evidence, then, that the natural or acquired refractoriness of certain rats to the tumor is due to the presence of such antibodies as are responsible for the ordinary anti-bacterial immunities. It seemed possible, however, that the blood of such refractory animals might still have some preventive or inhibiting effect on tumor growth, and a number of experiments have been undertaken to determine this point. Some of these experiments were done on the supposition that all races of rats would prove equally susceptible to the tumor under normal conditions and therefore do not answer the question as put. But the experiments on rats that were later, from the controls, found to be insusceptible, are of distinct interest, as will be evident. All of the animals in the following series were of similar weight (50–75 grams).

EXPERIMENT II.

The effect of the previous injection of blood in insusceptible animals followed by cancer. Nine rats from dealer "H" were treated as follows:

Lot 1. 3 rats. No treatment (Controls).
Lot 2. 3 rats. Given each 1.5 cubic centimeters defibrinated blood of normally susceptible rats.
Lot 3. 3 rats. Given each 1.5 cubic centimeters defibrinated blood of refractory rats.*

Nine days later all were implanted with pieces from the same tumor with the following result:

Lot 1. No tumors grew.
Lot 2. 1 tumor grew.
Lot 3. 2 tumors grew.

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*That is, rats which had failed to grow the tumor and had been reimplanted once or twice with the tumor.

It is evident from the controls (Lot 1) that these rats are normally insusceptible to the tumor. The previous injection of blood of susceptible rats and particularly of refractory rats would seem to facilitate growth of the tumor.

EXPERIMENT III.

The effect of the simultaneous inoculation of various bloods and cancer in insusceptible animals.

Twelve rats, source "P," were given each 1.5 cubic centimeters of the blood indicated on the same day on which they received an implantation of cancer. The blood was injected on the side opposite to the cancer implantation.

Lot 1. 3 rats. Cancer alone (Control). 1 tumor.
Lot 2. 3 rats. Cancer + blood normally susceptible rats (C). No tumors.
Lot 3. 3 rats. Cancer + blood normally insusceptible rats. 2 tumors.
Lot 4. 3 rats. Cancer + blood refractory rats. 3 tumors.

It would certainly seem indicated from these experiments, particularly the second, that the blood of insusceptible or of refractory animals injected previously, or at the same time as the implantation of the cancer in normally insusceptible animals, would lead to an increase in the number of takes. This is controlled both by the non-injection of blood and the injection of normal susceptible blood.

The results with relatively susceptible rats "L" and similar bloods are quite different.

EXPERIMENT IV.

Simultaneous injection of blood and cancer in moderately susceptible rats.

a. 4 rats given cancer alone. 2 tumors.
4 rats given cancer + susceptible blood. 4 tumors.
4 rats given cancer + refractory blood. 1 tumor.
b. 5 rats given cancer alone. 3 tumors.
5 rats given cancer + susceptible blood. 2 tumors.
5 rats given cancer + refractory blood. 4 tumors.

Obviously no conclusions can be drawn from these latter experiments. The one point of interest seems to be the apparent stimulating effect to tumor growth in insusceptible animals by the injection of the blood of refractory animals.

These facts all go to show that the immunity or refractory condition in animals to cancer is not explicable along the lines of ordinary anti-bacterial immunity.

It is evident that this resistance is dependable on very slight variations when we consider that mere change of locality may cause an insusceptible animal to become susceptible (Haaland). This led me to consider a factor that has seemed to be of some importance in another form of " iso-immunity." I[20] found that the isoagglutination of human blood was accompanied by certain differences in the tonicity of the bloods in question. For instance, if the blood serum of "A" agglutinates the red blood cells of "B" it will be found that the corpuscles of A and B differ in the extent to which they undergo dissolution in salt solutions of various concentrations. A careful comparison of the defibrinated blood of several normal, refractory, and cancer-bearing rats failed to show any consistent variations in their resistance to hypotonic solutions of sodium chloride.

Ehrlich has offered an explanation of cancer immunity which for a time at least seemed rather plausible. Although the successful transplantation of the tumor of one species in an animal of another species is usually impossible, Ehrlich found that when a mouse tumor was transplanted in a rat it would grow for a period of, say, eight days and then begin to retrograde. This mouse tumor that has grown in the rat " takes " perfectly well when brought back into the mouse and may subsequently be grown for a similarly brief period in another rat. And again Ehrlich states, on the basis of numerous experiments, that a mouse bearing one tumor will not grow a second implanted tumor. Largely on the basis of these two sets of observations he has offered his theory of " atreptic immunity (*α* privative and *τρέφω* = I nourish). According to this hypothesis the growth of a tumor depends on the presence of certain "X" substances, which directly or indirectly furnish a specific nourishment for cancer cells. The mouse tumor will grow in a rat as long as the "X" substance brought over with the tumor lasts; these substances are replenished on second growth in a mouse and the tumor may then grow in another rat for a time. Similarly the failure of a secondary implanted tumor to grow in Ehrlich's cases would be referable to a monopoly of the "X" substances by the original tumor.

A consideration of this atreptic theory has led to numerous results of interest. In the first place Ehrlich's general assertion that a second implanted tumor will never grow has been entirely disproved. Bridré found that second mouse tumors grew perfectly well, and both Lewin and Flexner and Jobling get a growth of second tumors in rats. Loeb regards a tumor-bearing animal as even more susceptible to tumors than a normal one, with which statement Gierke agrees. Simultaneously inoculated tumors both grow equally well (Bridré, Gierke, and myself). Schone[21] found that if he removed a growing tumor and a week or two later implanted a second one it invariably grew. Sticker divided the phases of tumor growth into two distinct periods, in the first, during which there are no metastases, a second implantation grows only when the first tumor is removed. During the second period (metastatic) a second tumor will grow as well as in a normal animal. Similarly, Flexner and Jobling found that their second implant would grow in a larger number of cases during this second period.

I had, independently of Schone's observation, already made some experiments as to the effect of tumor removal on the growth of a second tumor. In my own experiments I have implanted a second tumor on the opposite side immediately after removal of the primary tumor. As the age of growth of the first tumor is of some importance it has been included:

TABLE II.

On the growth of a second tumor implanted at once after removal of a primary tumor.

Rat No.	Age of Tumor Removed.	Second Tumor.
1	16 days.	No growth.
2	28 "	" "
3	28 "	" "
4	30 "	" "
5	30 "	" "
6	30 "	" "
7	30 "	Growth.
8	30 "	Growth.

It would seem, then, as if the second implanted tumor fails to grow after removal of the first tumor in the majority of instances up to thirty days. The probable relation of the presence or absence of metastases to these results can be determined only on fuller evidence as to growth of the second tumor after longer periods of growth of the first.

Of far greater interest is the result of experiments which were designed in the first place to control these results of reimplantation after removal. In order to control the effect of removal as compared with non-removal of the original tumor, animals were left with the original tumor and a second implanted. In a few of these animals the second tumor grew, but in most it did not, and in these latter animals it was found not only that the second tumor failed to grow but that in many instances the original tumor disappeared.

EXPERIMENT V.

Four rats in a given experiment showed tumors that had been implanted twenty-eight days previously on the left side. The largest of these tumors measured one centimeter in diameter. There were also on hand four normal animals of the same strain that had never been inoculated.

All eight animals were implanted with tumor F. F. 1 on the right side with the following result:

4 normal rats.	Tumors grew in 3.
4 tumor rats.	Second tumor grew and original continued to grow in 2. Second tumor did not grow and original tumor disappeared in 2.

The controls show that the second tumor was viable, inasmuch as three of the four animals gave positive growths. The growth of the second tumor in two of the tumor rats further evidences this fact.

It is unfortunate, as will later appear, that no histological material was saved from these two animals. The point of greatest interest, however, is the fact that in two tumor animals in which the second tumor failed to grow the first tumor actually disappeared.

A more striking instance of the disappearance of the original tumor apparently due to the implantation of a second tumor is shown in

EXPERIMENT VI.

Ten rats of a given lot that had been implanted with tumor thirty days previously showed tumors of from one to two centimeters in size, and were reimplanted with tumor on the other side of the belly. The original tumor was removed in five of these animals just before the second implant; as already noted (Table II.) in two of these animals the second tumor grew. In the other five the original tumor was left and the second implantation gave the following result:

In one animal the second tumor grew and the first tumor continued to grow.
In four animals the second tumor failed to grow and the first tumor disappeared.

These experiments indicate very strongly that the implanting of a second tumor may result in many instances in disappearance of the original tumor as well as in a failure of the second to grow. As this result was unexpected the experiments were none of them carried out in such a way as to make this "cure through vaccination" appear to the best advantage. Certain controls were omitted. It may be questioned, for example, as to whether or not the original tumors might not have disappeared spontaneously. The data at hand in the form of histories of individual animals make it seem most probable that tumors marked as "positive" on the fourth week rarely disappear; and yet experiments to show "cure" should unquestionably include such control animals. In later experiments a careful histological control of the animals in which the original tumor entirely failed to disappear renders it very evident moreover that even in such animals a degree of "cure" had been effected by reimplanting the tumor.

EXPERIMENT VII.

On July 21st forty rats from dealer "L," weighing on an average fifty grams each, were given implantations of tumor F. F. 51 on the left side. On the twenty-first day there were thirteen of these animals with definite tumors that were then divided into two lots:

Lot 1. Five animals with the *smallest* tumors.
Lot 2. Eight animals with larger tumors.

The animals of Lot 1 received no further treatment. The animals of Lot 2 received reinoculation of tumor on the right side on the twenty-first day and on the back on the twenty-fourth day.

The resulting growths were as follows:

Lot 1.
1. Died 80th day and was partially eaten.
2. Died 90th day. No examination possible; eaten.
3. Killed 120 days. Tumor measures 2.5 x 2 centimeters and is firm with living periphery. No metastases.
4. Killed 120 days. Tumor measures 2 x 2 centimeters and is firm and living.
5. Killed 140 days. Tumor 1.5 x 1 centimeters, firm. Histologically it resembles the usual good growing tumor with numerous mitoses. (See Fig. 1.)

Lot 2. Nos. 1, 2, and 3 died shortly after reinoculation and were eaten so that no examination was possible.

Nos. 4, 5, and 6. By the sixtieth day the original tumor had disappeared and did not recur subsequently. The reimplanted tumors failed to grow.

7. Died on 65th day. No apparent cause. The original tumor measures 1.5 centimeters in diameter and is yellowish white in color. Histologically the nodule is composed of fibrous tissue containing a few strands of epithelial cells about the periphery. (See Fig. 3.)
8. Killed on 95th day. All three tumors are present and do not differ from the ordinary type except in being more necrotic than usual.

Allowing for those animals in which examination was impossible through accident the experiment may be summarized:

In all three controls the tumor grew.

Of five treated animals the tumor disappeared entirely in three and was nearly replaced by connective tissue in a fourth when it died; in none of these four did the second or third tumor grow. In the fifth animal both the second and third tumors grew and the first failed to disappear.

EXPERIMENT VIII.

Sixteeen "L" rats were given tumor F. F. 28 on July 9th. On the thirty-third day nine of these animals showed definite tumors and were divided into lots and treated as follows:

Lot 1. Three animals with the smallest tumors (largest 1.6 x .7 centimeters and smallest 1.3 x .8) were given no further treatment.

1. Died 37th day. No examination possible.
2. Killed 90th day. Large tumor with growing periphery and necrotic center. No metastases.
3. Tumor failed to grow.

Lot 2. Six animals with largest tumors (varying in size from 2.8 x 1 centimeters to 1 x 4 centimeters) were given tumor F. F. 46 on right side and three days later tumor R. 2 on back.

Nos. 1 and 2. On the 90th day and subsequently no tumors were present.

3. Killed 90th day. There are the remains of the first and second tumors which are necrotic. Histologically the first tumor shows granulation tissue but no epithelial cells. In the second tumor there are the remnants of a few necrotic epithelial cells.
4. Killed 90th day. Two necrotic areas mark first and second tumors. The few remaining tumor cells are necrotic and vacuolated with pyknotic nuclei. There is no increase of connective tissue. (See Fig. 2.)
5. Killed 90th day. Third tumor alone evident. Histologically it shows degenerated epithelial cells and granulation tissue.
6. Killed 90th day. Remains of two tumors present. In the first there is increased connective tissue about the periphery and thin strands of epithelial cells. The other tumor is similar.

In this experiment the tumor failed to grow in one of the two controls which survived.

In two of six treated animals no tumors were left on the ninetieth day. In the remaining four, killed on the ninetieth day, the epithelial cells were degenerating and had all but disappeared in three, and were largely replaced by connective tissue in one.

The cumulative evidence from these four experiments, each of which is fairly conclusive in itself, would seem to show very definitely that the reimplanting of a tumor in a tumor-bearing animal twenty-one to thirty-three days after the first tumor will usually lead to a disappearance of the original tumor and a failure of the implanted tumor or tumors to grow. Thus, in twenty treated animals the tumor disappeared in twelve, had entirely degenerated or been replaced by connective tissue in four, and failed to be affected by the reimplantation in four cases only; in these latter cases it is to be noted that the reimplanted tumors also grew.

It is only fair to describe at once the only other experiments of this nature that have been performed and which failed to give as marked evidence of cure after reimplantation for reasons that are not quite evident.

EXPERIMENT IX.

Thirty-four "L" rats were given tumors on left side, and forty-one days later sixteen showed positive tumors. Six animals showing smallest tumors were left untreated. The tumor continued to grow in all. They were killed at intervals from the one hundred and ninth to the one hundred and fifty-sixth day, and four of the six showed metastases. The ten remaining tumor rats were given tumors on the forty-first and on the fifty-second day. When killed they showed the following conditions:

1. Killed 110th day. 3 tumors. Metastases.
2. Died 112th day. 1 tumor. No metastases.
3. Killed 112th day. 2 tumors. Metastases.
4. Killed 112th day. 2 tumors. No metastases.
5. Killed 112th day. 2 tumors. No metastases.
6. Killed 141st day. 2 tumors. No metastases.
7. Killed 143d day. 2 tumors. No metastases.
8. Killed 143d day. 2 tumors. No metastases.
9. Killed 143d day. 2 tumors. No metastases.
10. Killed 143d day. Small original tumor. No metastases.

The histology of the tumors from 1 to 6 inclusive resembled that of the controls. The tumors on the last four animals, however, showed a distinct increase in connective tissue over the tumors in the control rats. It is also of distinct importance to note that metastases were present in only two of the ten treated animals whereas they were shown by four or six controls. The late period at which the treatment was begun in this experiment (forty-one days) is also a factor of probable importance. Whereas this experiment cannot be used as a definite proof of the curative value of reimplantation it is certainly indicative of an attempt at cure.

EXPERIMENT X.

Fifteen young "L" rats, average weight seventy grams, were given tumor from F. G. 28 on left side October 26. Of these, six showed distinct tumors of approximately the same size on the twenty-fourth day and were divided into two equal lots and treated as follows:

Lot 1. Controls. No further treatment.
Lot 2. Given tumor from F. G. 29 on right side.

They were all chloroformed on the fifty-eighth day and showed the following picture:

Lot 1:
1. Firm living tumor. Metastases in lungs.
2. Tumor 2 x 2.5. No metastases.
3. Tumor 3 x 2.5. Metastases in lungs.

Lot 2:
1. Died before this date. No examination.
2. No tumors. Pin head connective tissue nodule indicates original tumor. No metastases.
3. Original tumor shows considerable amount of living tissue. No second tumor. No metastases in lungs. Lymph node shows in growth of tumor.

Three controls were positive. One animal of two treated was cured, the other showed no distinct evidence of any reaction to implantation.

EXPERIMENT XI.

On October 24th sixteen young "L" rats, weighing on an average seventy grams, were given tumor from F. G. 94 on the left side. Twenty-four days later seven of these animals showed positive tumors and were divided into two lots as follows:

Lot 1. 3 rats with tumors averaging 1.5 x 1.2 centimeters (largest 1.8 x 1.6, smallest 1.2 x 1) were left untreated as controls.

Lot 2. 4 rats with tumors averaging 2.2 x 1.3 centimeters (largest 3 x 1.5 centimeters, smallest 1.9 x 9) were given tumor F. G. 29 on right side.

One treated animal died in a few days. The remaining rats were chloroformed on the sixtieth day with the following findings:

Lot 1:

1. Firm growing tumor. Metastases in lungs.
2. Small firm tumor. No metastases.
3. Large necrosed tumor. No metastases.

Lot 2:

1. First tumor necrotic; second 1.5 x 1 centimeters. No metastases.
2. First tumor 3 x 2.5; second 3 x 1.5 centimeters. Metastases in lungs.
3. First tumor 3.5 x 2.5; second 2.5 x 1.5 centimeters. Metastases in lungs.

Histologically there were no differences between the tumors of treated and untreated animals. There is then no evidence of cure in this experiment.

Reinoculation of tumors in tumor-bearing mice has, of course, been tried repeatedly since Ehrlich's statement that a second tumor would not grow. Those writers who have considered this question have, however, failed to more than touch on the possibility of a cure of the first tumor through implantation. That there may be a stage (pre-metastatic period of Sticker) in the growth of a tumor during which reimplantation will lead to resorption of the original as a rule, has apparently escaped their notice. Bridré, to be sure, notes in a few sentences an apparent cure which followed injections of fresh tumor maceration in one or two animals, and Gierke[26] mentions in a footnote an unpublished communication of Bashford as to the possibility of rendering tumor animals immune by treatment with badly growing spontaneous tumors. The general experience has been, however, that a tumor would take better in a tumor-animal than in a normal one. Such a statement doubtless depends on experiments performed during the metastatic period.

The suggestion has already been made as to the possible effect of a metastatic period on this "cure by vaccination." According to Sticker during the first "non-metastatic period" no second implant will take, and it would also appear from my results that during this period this second implant may lead to cure. We may regard this first period as present as a period of adequate defence to invasion on the part of the host. It is of great interest to note that in human beings suffering from cancer, and often they were very early cases, that a specific "reaction of fixation" between the patient's blood serum and cancer emulsion has been found both by Ludke,[22] and more recently by Simon and Thomas.[23] As already mentioned, both Michaelis and I have found no such reaction with the serum of refractory rats. A summary of some experiments, performed and controlled as already detailed, with the serum of cancer-bearing rats with note as to the presence or absence of metastases is of interest.

TABLE III.

Reaction of fixation experiments with the serum of cancer rats.

Rat No.	Age of Cancer.	Metastases.	Result.
F. E. 4	16 days.	o	o
F. G. 88	....	o	o
F. E. 1	34 days.	o	+
F. G. 89	....	o	o
F. E. 17	34 days.	o	o
F. G. 28	....	o	+
F. E. 9	37 days.	+ +	o
F. E. 2	37 "	+	o
F. D. 15	37 "	+ +	o
F. G. 92	156 "	+	o

Thus in two cancer animals that had no metastases a reaction of fixation was present. No such reaction was obtained during the metastatic period. The presence of an active resistance on the part of the tumor animal during the premetastatic period as indicated by a reaction of fixation, and an absence of such resistance during the metastatic period, would explain the reason for a cure when the animals are "vaccinated" with a new tumor during the first few weeks as well as why the second tumor grows during a later metastatic period.

SUMMARY AND CONCLUSIONS.

In the majority of my experiments in transmissible tumors I have employed the Flexner-Jobling tumor of the rat. As it occurred originally this tumor was described as a sarcoma, but later was mentioned by the authors in question as having changed to a carcinoma, which was its character when it came into my hands. It has showed no marked histological changes during the eight generations through which I have cultivated it.

The susceptibility of white rats obtained from various sources to this tumor varies remarkably. Whereas the animals from one source gave one hundred per cent of "takes" the next most susceptible strain gave only fifty per cent of positive results.

Following successful subcutaneous implantation in the region of the axilla, metastases occur regularly in the lungs and only very rarely in the adjacent lymph nodes. The rapidity with which metastases occur would seem to depend on the susceptibility of the strain of rats employed and in the most susceptible strain would seem to occur at a rather definite period after implantation.

Successive transplantations from lung metastases in the most susceptible strain apparently lead to an increased virulence in the tumor, as indicated by a rapidity in occurrence and increased number of metastases, as well as by a distinct increase of the epithelial constituents of the tumor over the stroma.

Animals that have failed to take the tumor on first inoculation are very seldom susceptible to a second or third implantation. The blood serum of animals rendered refractory by several implantations, as well as the serum of animals of a normally insusceptible strain, gives no fixation of alexin with an extract of the tumor employed. No distinct preventive effect is evident on simultaneous injection of such "refractory" blood and cancer in animals that are naturally susceptible. This refractory blood when injected previous to or at the same time as implantation of cancer in normally insusceptible rats would seem definitely to increase the percentage of takes.

The bloods of normal, of cancer-bearing, and of refractory rats show no difference in tonicity as measured by the resistance of their respective blood corpuscles to hypotonic solutions of sodium chloride.

Following the removal of a tumor that has been growing for from sixteen to thirty days a second implantation of tumor usually fails to give a positive result. A few animals on the thirtieth day gave a positive second tumor. This period extending to the thirtieth day corresponds rather closely to the limit of the period during which no metastases are found (Sticker).

When the primary growing tumor is left and a second tumor implanted during the "pre-metastatic" period, in many instances the second tumor not only fails to grow but the first tumor entirely disappears. In cases in which resorption of the original tumor was incomplete when the animal was killed, either from lack of sufficient time for the effecting of a complete cure or in a case of incomplete cure (in which case the second tumor may grow), the original tumor shows distinct histological changes. These changes are evident either in the form of degeneration and vacuolization of the protoplasm of the cancer cells with pyknotic changes in their nuclei, or else by the occurrence of an enormous increase of connective tissue which compresses the remaining strands of epithelial cells. No such changes have been found in non-treated tumors.

A reaction of fixation has been found between cancer extract and the blood serum of a few cancer-bearing rats during the pre-metastatic period, but never during the metastatic period. The pre-metastatic period, then, would seem to be characterized by an active defence on the part of the animal as indicated by the presence of antibodies. During this reactive period, reimplantation will increase the resistance to such an extent that the original tumor is destroyed. When this period is passed metastases occur and a second implanted tumor grows.

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DESCRIPTION OF PLATE IV.

FIG. 1. — Ordinary tumor growing one hundred and forty days in a non-treated rat (compare with Fig. 3). Normal relations of cancer cells and stroma.

FIG. 2. — Original tumor from rat that had received reimplantations on thirty-third and thirty-sixth day. Killed on ninetieth day. Epithelial cells show degenerated and vacuolated cytoplasm and pyknotic nuclei.

FIG. 3. — Transplant from same tumor as rat in Fig. 1 in another rat of same strain that was treated with a second and third implantation. Sixty-five days after implantation of tumor (compare with Fig. 1). Marked increase of connective tissue stroma with compressing of columns of epithelial cells.

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240 Longwood Avenue,
Boston, Massachusetts, U.S.A.

MAY 24 1909
14,007

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VOLUME XX., No. 3
(New Series, Vol. XV.)

APRIL, 1909

Whole No. 113

THE JOURNAL OF MEDICAL RESEARCH

Continuation of the
JOURNAL OF THE BOSTON SOCIETY OF MEDICAL SCIENCES

EDITED BY
HAROLD C. ERNST, M.D.

EDITORIAL OFFICE: 240 LONGWOOD AVENUE
BOSTON, MASS., U.S.A.

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Journal of Medical Research.

(New Series, Volume XV.)

Vol. XX., No. 3. APRIL, 1909. Whole No. 113.

THE QUANTITATIVE RELATIONS OF ANTIGEN, AMBOCEPTOR, AND COMPLEMENT IN THE ESTIMATION OF HEMOLYSIS.*

Samuel S. Kneass, M.D., and Joseph S. Evans, M.D.

(*From the William Pepper Laboratory of Clinical Medicine, University of Pennsylvania.*)

The method of diagnosis of specific amboceptors in sera by means of a deviation or absorption of a complement is too well known to need more than a passing description.

The heated serum of an animal immunized by repeated injections of an antigen, *i.e.*, bacteria, or cell elements, liver cells, red blood corpuscles, of an animal of another species, contains a specific thermostabile amboceptor which, united on the one hand with the given antigen and with a fresh unheated non-specific normal guinea-pig's serum on the other, will bring about a destruction of these cells or bacteria, both in vivo and in vitro. If, to such a system, composed of a specific antigen, as a prepared emulsion of a syphilitic liver (containing the spirochetæ), a specific heated serum from a syphilitic, and a complement, fresh unheated normal guinea-pig's serum, we add a combination of inactivated hemolytic serum and a hemolytic antigen — erythrocytes, no destruction of the red cells should occur, because the necessary complement is not present, having been deviated to or absorbed by the reaction in the syphilitic primary system. If the first serum be not specific, then no combination with the syphilitic antigen takes place, the complement remains free to act with the members of the hemolytic series, and a destruction of the erythrocytes or hemolysis occurs.

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* Received for publication Feb. 2, 1909.

This reaction has been cited since the application of the method in the diagnosis of syphilis has been the most interesting of the many investigations along this line, and the one which, if practicable, promises much in the field of clinical medicine. The results of such experiments, however, while indicating the possible value as an aid to diagnosis, show that certain important principles have been overlooked which might either vitiate or confirm the conclusions.

Five substances are necessary in such a reaction: a specific antigen, a specific serum, a complement, a hemolytic serum, and a hemolytic antigen. It was the purpose of our experiments to determine, if possible, what quantitative relations might exist between these several substances and thus obtain a knowledge of a standard with which to compare the strength of a given serum in specific amboceptor or in complement. Does a given antigen anchor a determinable ratio of specific immune body and, if such be the case, can this ratio and the haptophore activity of such immune serum be calculated in terms of the complement? Is, again, the guinea-pig or other normal serum sufficiently uniform in its complement content to be used as a unit of measurement?

These questions were some of those, arising at the beginning of the work, that showed us that the first problems at issue were the activity and uniformity of the complement and the quantitative relationship of such complement and the members of the hemolytic series, since in any foreign series, *e.g.*, the syphilitic, the specific amboceptor content of a serum must vary according to the degree of reaction on the part of the individual, and in the preparation of the antigen there must be some variability because of the unknown character of the substance with which we are dealing.

In the hemolytic series we are dealing with quantities of three substances, two of which are unknown. The number of erythrocytes in suspension and the amount of hemoglobin in combination or, when hemolysis occurs, in solution, can be determined; and if there be any definite relation between amboceptor, blood cells, and complement this can be expressed in terms of the known quantity — the number of red cells hemolyzed. Having found the amount of complement necessary to unite with a certain amount of amboceptor and hemolyze a given number of erythrocytes, we can add these quantities, or multiples of such, of the hemolytic amboceptor and antigen to a specific series, to which has already been added the required quantity of complement: and thus the quantity of complement absorbed by the first series can be calculated by subtracting from the total complement present the amount remaining active and producing hemolysis in the hemolytic series. Varying the quantities of specific serum and antigen, we should be able to arrive at some conclusions as to the quantitative relations of these latter.

To determine such possible numerical relations between the members of the hemolytic system was the purpose of these experiments, and the methods and results are detailed in this paper.

Preparation of antigen. — We made use of the blood corpuscles of the sheep. The animal was bled from the external jugular vein through a large hypodermic needle and the blood collected and shaken in a sterilized aspirating bottle containing copper wire or glass tubing. This defibrinated blood was then counted by the Thoma-Zeiss instrument, and a hemoglobin estimation made as described below, as a control for dilution, the object being to have a suspension containing about one million corpuscles to the cubic millimeter. Of this blood a certain quantity (generally about twenty cubic centimeters) was pipetted into tubes and centrifugalized. The clear fluid was decanted off, the deposited cells very carefully shaken up with one per cent NaCl solution and the mixture centrifugalized. This process was repeated at least four times to remove any trace of serum. To these centrifugalized blood cells was added one per cent NaCl solution in such amount as to give a suspension containing one million cells to the cubic millimeter. It was found that, in practice, it was unnecessary to have this exact quantity in suspension, as the estimations made from the number found to be present could be reduced by calculation to the basis of one million. In fact, in a number of experiments we used a suspension containing only four hundred thousand, and the quantity of amboceptor and complement found requisite to completely hemolyze them was multiplied by two and one-half. The final suspension for experimental use was carefully counted a number of times, and a number of hemoglobin estimations were made; these two should, of course, both show the same dilution of the original counts.

Preparation of amboceptor.—The hemolytic serum was obtained from rabbits previously inoculated with the washed corpuscles of sheep. Five cubic centimeters of a twenty per cent suspension of such corpuscles in isotonic salt solution were injected intraperitoneally into rabbits on each fourth day for four doses. One week after the final injection the animals were bled from the auricular vein, the serum being separated and divided into tubes of two cubic centimeters each. These were heated at 50° C. for thirty minutes, sealed with paraffin, and stored at refrigerator temperature. In this way we were enabled to carry on a series of experiments with the same amboceptor containing serum. Throughout our experiments we employed ten animals and found that the above method of immunization produced a constant amboceptor value in their various sera.

Preparation of complement.—The complement used was that contained in the serum of the normal guinea-pig. Blood was obtained by severing the carotids directly into sterile centrifugalizing tubes. After coagulation the clot was carefully separated from the tube wall and the tube placed in the ice-chest for a few hours. It was then centrifugalized, and the serum decanted and placed on ice until used. Fresh serum, twenty-four hours old, was usually employed. In a few cases, three days old complement was used and found to be of full activity, but some six days old had diminished in activating power. The serum was generally diluted with ten parts of one per cent salt solution and this was added in requisite amounts to the system.

Method of making mixtures. — In the earlier experiments we made use of pipettes that gave a certain number of drops to the cubic centimeter, but in the later ones, pipettes graduated to 1/100 cubic centimeter and 1/1,000 cubic centimeter reduced the error of measurement to a minimum. The total quantities of sera to be used were diluted with salt solution; the amboceptor to 1/100 and 1/500, and the complement to 1/10 and 1/100. The desired amount of diluted immune serum was first added, then the diluted complemental serum and the total made up to one cubic centimeter with saline solution. To this was then added one cubic centimeter of the blood suspension. The series of tubes was then placed at a temperature of 37° C. for one hour and then at ice temperature for at least two hours, after which they either remained on ice over night for hemoglobin estimation the next morning, or were immediately centrifugalized and the Fleischl readings taken.

Estimation of hemolysis. — At first the amount of hemolysis was determined by comparison with known dilutions of the blood suspension in use at the time. The difficulty here was the impossibility of comparing the various tubes, especially in the low dilutions, and also the necessity for carefully making up a new scale with each experiment. An attempt was made with an artificially prepared color scale, but the first objection mentioned obtained here also. The use of the Fleischl Hemoglobinometer gave a more accurate and certainly a more convenient method for the determination of the hemoglobin. With the original defibrinated blood the small Fleischl pipette can be used, but in estimating slight amounts of hemolysis a much larger measure is necessary, and our estimations are based on readings made with one hundred cubic millimeters of the liquid examined. The relations of readings obtained by these two measures may be readily calculated. The instrument used was No. 7.85.

Hence a reading of 50 with 7.85 cubic millimeters would correspond with $7.85 : 100 = 50 : 6.40$ obtained with one hundred cubic millimeters. This calculation can be dispensed with, as the reading with 7.85 cubic millimeters denotes the percentage of the normal quantity of hemoglobin in one hundred cubic centimeters of human blood (ca. 12.75 g.), and therefore, with one hundred cubic millimeters, gives the actual amount of hemoglobin in one hundred cubic centimeters. Thus a reading of 80 with one hundred cubic millimeters means that there is .8 g. in one hundred cubic centimeters.

$$100 : 7.85 = .80 : .0628 \times 12.75 = .80.$$

In some cases the number of erythrocytes hemolyzed was found by counting those remaining unchanged after partial hemolysis, but generally was estimated from the percentage of hemolysis, as described above, the two phenomena being proportionate.

The following tables indicate the result of our experiments. The symbols used are explained as follows:

S = Sheep's blood at time of bleeding.
R = Rabbit inoculated with sheep's blood.
A = Amboceptor obtained from the immunized rabbits.
C = Complement obtained from guinea-pig.
B = Suspension of erythrocytes of the sheep.

The numerals indicate the various bleedings, blood mixtures, and animals used during the experiments.

EXPERIMENT I. 6/22/07.

No.	Ao (Active).	B1.	NaCl (.85%).	Water.	Reaction.
1.	.1 cc.	1 cc.	1 cc.	....	Hemolysis.
2.	.1 "	" "	" "	....	"
3.	.01 "	" "	" "	....	No hemolysis.
4.	.01 "	" "	" "	....	" "
5.	.001 "	" "	" "	....	" "
6.	.001 "	" "	" "	....	" "
7.	.0001 "	" "	" "	....	" "
8.	.0001 "	" "	" "	....	" "
9.	....	" "	" "	....	" "
10.	....	" "	" "	1 cc.	Complete hemolysis.
11. To tube No. 3 was added .08 cc. making .09 cc. Ao.				....	Hemolysis.
12. " " " 4 " " .026 " " .036 " "				....	"
13. " " " 5 " " .053 " " .054 " "				....	"
14. " " " 6 " " .066 " " .067 " "				....	"

Ao = R2 injected with 5 cc. and 35 cc. intraperitoneally of B1.

B1 = S1 bled 6/17/07, 40 cc. being taken from external jugular into sterile bottles defibrinated and centrifugalized. Corpuscles washed in .85% NaCl four times and diluted with twenty volumes NaCl. Estimated erythrocyte content about $.5 \times 10^6$ per cm. Hemolysis caused by something between .07 and .09 cc. Ao.

EXPERIMENT II. 7/11/07.

No.	A1 (Active).	B3.	NaCl (.85%).	Water.	Reaction.
1................	.2 cc.	1 cc.	1 cc.	....	Complete hemolysis.
2.	.1 "	" "	" "	....	" "
3................	.04 "	" "	" "	....	Slight "
4................	.02 "	" "	" "	....	No "
5................	.01 "	" "	" "	....	" "
6................	.004 "	" "	" "	....	" "
7................	.002 "	" "	" "	....	" "
8................	.001 "	" "	" "	....	" "
9................	.0004 "	" "	" "	....	" "
10................	....	" "	2 "	1 cc.	" "
11................	....	" "	....	" "	Complete "
				Normal Sheep's Serum.	
12................	....	" "	1 cc.	1 cc.	No "
13. To tube No. 4 was added .5 cc. sheep's serum.				....	" "
14. " " " 5 " " .1 " complement C1.				....	Complete "
15. " " " 6 " " " " " "				....	" "
16. " " " 7 " " " " " "				....	" "
17. " " " 8 " " " " " "				....	" "
18. " " " 9 " " " " " "				....	Incomplete "
19. " " " 12 " " .2 " inactivated A1.				....	No "

A = R3 injected intraperitoneally with B2, 4 cc., 6/26/07; 5 cc., 6/28/07; 4 cc., 7/1/07; 5 cc., 7/3/07; and killed 7/10/07.
B2 = S2, 6/20/07.
B3 = S3, 7/10/7. Suspension made in .85% NaCl, containing about .5 × 10^8 Erythrocytes per cm.
C1 = Guinea-pig's serum obtained 7/11/07, three hours old.

This experiment shows, as did Experiment I., that complete hemolysis of B1 is produced by about .08 cubic centimeter of active hemolytic sera (A0 and A1), which indicates that this amount of serum contains a sufficient quantity of amboceptor and complement for complete anchorage upon all the erythrocytes in suspension. As .001 cubic centimeter R2, in conjunction with .1 cubic centimeter C1, however, produced hemolysis with complete solution of the corpuscles, we see that in .08 cubic centimeter R2 there is a great excess of amboceptors, but the proper amount of complement. In later experiments we found that the amount of complement necessary to bring about complete hemolysis of one cubic centimeter red blood corpuscle suspension is .04 cubic centimeter; hence the .08 cubic centimeter active A0 and A1 must contain .04 cubic centimeter complement, or rather the quantity of complement contained in .04 cubic centimeter guinea-pig's serum. If we take the latter as a standard, then active A0 and A1 are fifty per cent strength of C. Sheep's serum contains no complement since .5 cubic centimeter with .02 cubic centimeter A1 produced no hemolysis.

Experiment III. 7/12/07.

No.	A1 (Inactive).	C1.	B3.	NaCl (.85%).	Reaction.
1......	.0004 cc.	.1 cc.	1 cc.	1 cc.	Marked hemolysis.
2......	....	" "	" "	" "	No "
3......	.0002 cc.	" "	" "	" "	25% "

A1 (inactive) = heated at 50° C. one hour.
C1 = 24 hours old.
B3 = 24 " "

Experiment IV. 7/12/07.

No.	A1 (Inactive).	C2.	B3.	NaCl (.85%).	Sheep's Serum.	Reaction.
1.......	.0004 cc.	.1 cc.	1 cc.	1 cc.	....	Maximum hemolysis.
2.......	" "	.06 "	" "	" "	....	" "
3.......	" "	.016 "	" "	" "	....	" "
4.......	....	.01 "	" "	" "	....	No hemolysis.
5.......	.0004 cc.	....	" "	" "	....	" "
6... ...	....	.01 cc.	" "	" "	.1 cc.	" "

C2 = Fresh, 7/12/07.

These two experiments confirmed the previous findings:

that sheep's serum produced no hemolysis of its own corpuscles and that .0004 cubic centimeter A1 in conjunction with complement marked but incomplete hemolysis. The maximum hemolysis with this amount of amboceptor was produced with .016 cubic centimeter complement as well as with .1 cubic centimeter, which indicates a quantitative relation between A and C. Incomplete hemolysis here, therefore, is due to the lack of amboceptor and not of complement. It is important, then, to determine the quantitative relation between the antigen and amboceptor. In order to do this it is necessary to have a standard suspension of the former, and, at the same time, a method of determining the degrees of hemolysis. At this point in our experiments we devised the scale of 1 to 20 previously described.

EXPERIMENT VI. 7/13/07.

No.	A1.	C2.	B3.	NaCl.	Scale of Hemolysis.
1	.0004 cc.	.02 cc.	1 cc.	1 cc.	6
2	" "	.02 "	" "	" "	6
3	" "	.002 "	" "	" "	1
4	" "	.004 "	" "	" "	1
5	" "	.006 "	" "	" "	1½–2
6	" "	.008 "	" "	" "	2–3
7	" "	.010 "	" "	" "	3½–4
8	" "	.012 "	" "	" "	4
9	" "	.014 "	" "	" "	5
10	" "	.016 "	" "	" "	6
11	" "	.018 "	" "	" "	6
12	" "	.018 "	" "	" "	6
13	" "	.020 "	" "	" "	6
14	" "	.05 "	" "	" "	6
15. To tube No. 3 was added .008 cc. C1 making total of .01 cc............					3
16. " " " 4 " " .016 " " " " " .02 "					6

A1 = R3 72 hours old.
C2 = 48 " "
B3 = 48 " "

Scale employed was a series of tubes marked 1 to 20 containing from .05 cc. to 1 cc. of the blood suspension used, hemolyzed and diluted up to 2 cc. with water.

The maximum hemolysis that can be produced with .0004 cubic centimeter A1 is 6 in scale of 20, or 6/20 of the total possible. If the amount of hemolysis is proportionate to the quantity of A, then complete solution of one cubic centimeter B3, reading 20 in the scale, should be caused with 20/6 × .0004 or .0013. If about $.5 \times 10^9$ erythrocytes are in the one cubic centimeter B3, then one cubic centimeter of "standard" blood suspension containing 1×10^9 should be hemolyzed with .0026 cubic centimeter A. That this is true we shall see hereafter.

Further we see that the minimum amount of C2 needed to activate the .0004 cubic centimeter A1 and to produce the maximum hemolysis is .016 cubic centimeter, or forty times the quantity of A1. All complement in excess of this produces no further effect, for the amboceptor is fully saturated.

C.	Ratio of C to total C.	This ratio × 6 = maximum H.	Color scale.
.016	1	6	6
.014	7/8	5¼	5
.012	3/4	4½	4
.010	5/8	3¾	3½–4
.008	1/2	3	2–3
.006	3/8	2¼	1½–2

This correspondence between the estimated and observed hemolysis, considering the difficulty of comparison of the various tubes, is sufficiently close to presume that with smaller amounts of C than that required to fully activate the .0004 cubic centimeter of A the hemolysis is proportionate to the quantity of C, and that the ratio of 40 to 1 still holds. For example, in number 8, .012 cubic centimeter C activated .0003 A; hence should hemolyze $\frac{.0003}{.0004}$ of the maximum amount or 4½ in the scale. If these relations exist, the exact hemolyzing power of any serum may be calculated from any one of the above tubes. Thus $4.5 : .012 = 20 : .053 \times 40 = .0013$ A necessary to hemolyze one cubic centimeter B3 or .0026 to hemolyze one cubic centimeter containing 1×10^9 erythrocytes per cubic centimeter. Although maximum hemolysis in Experiment VII. was produced by 1/40 A/C, still it was not complete hemolysis. This fact emphasized the importance of determining the quantitative relation of antigen and amboceptor. According to the above calculation .0013 cubic centimeter was completely anchored by $.5 \times 10^9$ erythrocytes per cubic centimeter.

EXPERIMENT VIII. 7/17/07.

No.	A1.	C3 (24 Hours Old).	B4.	NaCl (.85%).	Scale.	Calculated Scale.
1.....	.0004 cc.	.022 cc.	1 cc.	1 cc.	12	12
2.....	" "	.020 "	" "	" "	12	12
3.....	" "	.018 "	" "	" "	12	12
4.....	" "	.016 "	" "	" "	12	12
5.....	" "	.014 "	" "	" "	10+	10+
6....	" "	.012 "	" "	" "	8	8
7.....	" "	.010 "	" "	" "	....	
8.....	" "	.006 "	" "	" "	7	4
9.....	.001 "	.1 "	" "	" "	20	20
10.....	" "	.08 "	" "	" "	20	20
11.....	" "	.06 "	" "	" "	20	20
12.....	" "	.04 "	" "	" "	20	20
13.....	" "	.02 "	" "	" "	15	15
14.....	" "	.01 "	" "	" "	12	7.5
15.....	" "	.006 "	" "	" "	7−	4.5
16.....	.0002 "	.006 "	" "	" "	5+	4.5
17.....	" "	.002 "	" "	" "	2	1.5

In this experiment, at first sight, while the 40 to 1 ratio between the complement and amboceptor is apparent, the relation between the quantity of amboceptor and the amount of hemolysis seems to vary from what we have found in other experiments. This seeming variation from what we desire to regard as the general rule is explained by noting that while .001 cubic centimeter of amboceptor produces a complete hemolysis, this amount may not be the minimum quantity that can do so. If we calculate from tube No. 13, we have .02 cubic centimeter C activating .0005 cubic centimeter A and producing 15/20 total hemolysis. Hence .00066 cubic centimeter A should cause complete hemolysis. Further, .00066:20=.0004:12, which is the observed amount. The calculated scale numbers are based on this figure .0006.

In this case we dealt with an unknown blood suspension. If it should have contained 250,000 erythrocytes per cubic centimeter, this figure, .00066, would correspond to .00264 cubic centimeter of amboceptor for 1,000,000 erythrocytes per cubic centimeter. This showed us the necessity of using a known number of erythrocytes.

EXPERIMENT X. 10/2/07.

No.	A3.	C4.	B7.	NaCl (.85%).	Fleischl Reading.
1	.002 cc.	.1 cc.	1 cc.	1 cc.	36
2	“ “	.09 “	“ “	“ “	
3	“ “	.085 “	“ “	“ “	
4	“ “	.08 “	“ “	“ “	36
5	“ “	.075 “	“ “	“ “	33
6	“ “	.07 “	“ “	“ “	33
7	“ “	.065 “	“ “	“ “	
8	“ “	.06 “	“ “	“ “	
9	.001 “	.06 “	“ “	“ “	18
10	“ “	.055 “	“ “	“ “	
11	“ “	.05 “	“ “	“ “	
12	“ “	.045 “	“ “	“ “	18
13	“ “	.04 “	“ “	“ “	18
14	“ “	.038 “	“ “	“ “	16
15	“ “	.036 “	“ “	“ “	15
16	“ “	.034 “	“ “	“ “	15
17	“ “	.032 “	“ “	“ “	15
18	“ “	.03 “	“ “	“ “	
19	.0006 “	.035 “	“ “	“ “	12
20	“ “	.03 “	“ “	“ “	
21	“ “	.026 “	“ “	“ “	12
22	“ “	.025 “	“ “	“ “	12
23	“ “	.024 “	“ “	“ “	12
24	“ “	.022 “	“ “	“ “	11
25	“ “	.02 “	“ “	“ “	9
26	“ “	.018 “	“ “	“ “	7
27	.0002 “	.015 “	“ “	“ “	3¼

EXPERIMENT X. — *Continued.*

No.	A3.	C4.	B7.	NaCl (88%).	Fleischl Reading.
28	.0002 cc.	.012 cc.	1 cc.	1 cc.	3¼
29	" "	.009 "	" "	" "	3¼
30	" "	.008 "	" "	" "	
31	" "	.0076 "	" "	" "	
32	" "	.007 "	" "	" "	
33	" "	.0066 "	" "	" "	
34	" "	.006 "	" "	" "	
35	" "	.0056 "	" "	" "	1.5
36	" "	.005 "	" "	" "	
37	.4 "	....	" "	" "	0
38	....	....	" "	" "	0
39	....	.1 cc.	" "	" "	0
40	....	....	" "	Water. 1 cc.	40 +

A3 = R5 injected 9/18, 4 cc.
9/23, " "
9/25, 10 "
10/1, bled.

C4 = 24 hours old.

B7 = S5 (9/16). Hemoglobin (100 cm.) 84%.
Erythrocytes = .9 × 10^6 per cm.

The ratio 1/40 = a/c still holds.

The hemoglobin dissolved bears a constant ratio to the amount of amboceptor activated.

E.g.:

.002 cc. A + 40 times that amount of C = hemolysis 36.
.001 " " + " " " " " " = " 18.
.0006 " " + " " " " " " = " 12.
.0002 " " + " " " " " " = " 4.

36 (maximum H with .002 cc. A) : .002 = 42 (complete H) : .0023; *i.e.*, .0023 will hemolyze 1 cc. of B7 containing .9 × 10^9 erythrocytes, or .00258 cc. A will hemolyze a standard suspension containing 1 × 10^9 per cc.

Controls showed no hemolysis with A or C alone.

EXPERIMENT XII. 10/7/07.

No.	A3.	C4.	B9.	Saline (85%).	Fleischl.
1	.001 cc.	.08 cc.	1 cc.	1 cc.	16
2	“ “	.05 “	“ “	“ “	16
3	“ “	.045 “	“ “	“ “	16
4	“ “	.04 “	“ “	“ “	12
5	“ “	.03 “	“ “	“ “	10

A3 = 6 days old.
C4 = 6 “ “
B9 = Hb = 80%, erythrocytes, 1 × 10^6 per cm.

In this experiment the A and C were six days old. The maximum hemolysis is 16 or 4/10 of complete hemolysis. Therefore it will take .0025 cubic centimeter amboceptor to hemolyze one cubic centimeter of B9 (1 × 10^9 rbc.), and the amboceptor has undergone no change in power. The complement, however, is weaker, requiring more than forty times to activate the .001 cubic centimeter of amboceptor.

EXPERIMENT XVII. 10/22/07.

No.	A4 (Active).	C6.	B10.	Saline (88%).	Fleischl.
1	.2 cc.		1 cc.	1 cc.	24
2	.1 "		" "	" "	17
3	.06 "		" "	" "	7.5
4	.04 "		" "	" "	
5	.02 "		" "	" "	
6	.01 "		" "	" "	
7	.008 "		" "	" "	
8	.004 "		" "	" "	
9	.001 "		" "	" "	
10	.0005 "		" "	" "	
				Water.	
11			" "	1 cc.	25
12			.5 "	1.5 cc.	12
13.	To tube No. 5 is added .1 cc. C				24
14.	" " " 6 " " " " "				24
15.	" " " 7 " " " " "				24
16.	" " " 8 " " .16 " "				24
17.	" " " 9 " " .04 " "				24
18.	" " " 10 " " .02 " "				20 (?)
19.	" " " 4 " " " " "				20 (?)

A4 = 10/22/07, from R5, 4 injections more.
B10 = Hb = 48% from S7, 10/21.
Erythrocytes = $.4 \times 10^6$ per cm.
C6 = 10/22/07.

If .1 cubic centimeter active serum produces 17/24 of complete hemolysis, .14 cubic centimeter should produce complete hemolysis. If .06 cubic centimeter active serum produces 8/24, then .18 cubic centimeter should completely hemolyze the suspension B10.

The combinations in 18 and 19, .0005 cubic centimeter and .04 A4 + .02 C6 hemolyzing to 20, may be errors; if not, A4 hemolyzes at .0006 cubic centimeter or at .0015 cubic centimeter for a standard suspension, *i.e.*, 1×10^6 erythrocytes per centimeter. This does not correspond with other figures obtained from same A4. However, the several experiments with A4 showed great variations both in hemolytic power and in complementophilic action. This serum was the second obtained from a rabbit (R5) that had had a long period of injections, every three days for about a month. A third bleeding (A5) taken three weeks later showed more marked deviations still. It will be interesting to determine more definitely the effect on amboceptor of many repeated injections.

If .001 cubic centimeter A + .04 cubic centimeter C produce complete hemolysis, then in .18 cubic centimeter active A4, which completely hemolyzes, there must be the same quantity of complement that is in .04 cubic centimeter guinea-pig's serum. Active A4, then, has a 22.2 per cent complemental power, which differs greatly from the fifty per cent strength exhibited by A0.

EXPERIMENT XXI. 12/12/07.

No.	A7.	C9.	B14.	Saline (1%).	Fleischl.	Calculated.
1......	.001 cc.	.048 cc.	1 cc.	0 cc.	35	35
2......	" "	.044 "	" "	" "		
3......	" "	.040 "	" "	" "	35	35
4......	" "	.036 "	" "	" "	30+	31.5
5......	" "	.032 "	" "	" "	28	28
6......	.0008 "	.04 "	" "	" "	27	28
7......	" "	.036 "	" "	" "		
8......	" "	.032 "	" "	" "	27	28
9......	" "	.028 "	" "	" "	24	25
10......	" "	.024 "	" "	" "	20	21
11......	.0006 "	.032 "	" "	" "	20	21
12......	" "	.028 "	" "	" "		
13	" "	.024 "	" "	" "	20	21
14......	" "	.020 "	" "	" "	17	17.5
15......	" "	.016 "	" "	" "	12	14
16......	.2 "	....	" "	" "	0	
17......	....	.8 cc.	" "	" "	0	
18.. ...	....	....	" "	" "	0	

A7 = R6, 11/16 to 11/26, 4 injections.
12/10/07, bled.
B14 = Hb 35.
Erythrocytes .4 × 10^6 per cm.

This experiment further shows the correspondence of the experimental results and those based on the fact that .0025 cubic centimeter A7 should hemolyze one billion corpuscles in combination with forty times its amount of complement, or

$$1 \times 10^9 : .4 \times 10^9 . :: .0025 : .001.$$

EXPERIMENT XXVI. 12/19/07.

No.	Amb. 7.	C12.	B16.	Saline (ad.).	Fleischl.	Calculated.	
						.00225	.0025
1	.001 cc.	.048 cc.	1 cc.	2 cc.	22	22	18
2	" "	.042 "	" "	" "	22	22	
3	" "	.040 "	" "	" "	22	22	18
4	" "	.038 "	" "	" "	21	21	11
5	" "	.032 "	" "	" "	15	17	14.5
6	.0006 "	.030 "	" "	" "	12.5	13	11
7	" "	.026 "	" "	" "	12.5	13	
8	" "	.024 "	" "	" "	12.5	13	11
9	" "	.022 "	" "	" "	10	12	10
10	" "	.020 "	" "	" "	8	11	9
11	.001 "	....	" "	" "	0		
12	....	.04 cc.	" "	" "	1		
13	....	....	" "	" "	0		
14	....	....	" "	Water. 1 cc.	45		

A7 = R6, 4 injections (= 20 cc.), 11/16–11/23/07.
B16, 12/19/07; S11, 12/18/07.
Hb 90. Erythrocytes, 1 × 10^6.
C12, 12/18/07.

If .001 A produces 20/45 total hemolysis, .00225 A will cause complete hemolysis of one cubic centimeter standard blood suspension.

Calculating the theoretical Hb readings, on the basis that .0025 will completely hemolyze one cubic centimeter of B16, we obtain the following, subtracting for the color added with the C:

		Calculated.
22 — 1.2 = 20.8	21.	18.
22 — 1. = 21.		
22 — 1. = 21.		
21 — 1. =	20.	17.
15 — .8 =	14.2	14.5
12.5 — .75 = 11.75	11.8	11.
12.5 — .7 = 11.8		
12.5 — .6 = 11.9		
10.5 — .5 = 9.5	9.5	9.9
8. — .5 =	7.5	9.

EXPERIMENT XXXIII. 3/20/08.

No.	A9.	C21.	Fleischl ob5 less C color.	Calculated.	A9.	C21.	Fleischl less C color.	Calculated.
	1 cc. of B added and Saline to 2 cc.				Centrifugalized and washed four times in 1% saline solution and the following additions:			
1 ..	.002 cc.	.1 cc.	42.5	42	....	.04 cc.	0	0
2 ..	" "	" "	....	....	.001 cc.	.06 "	7.5	10
3 ..	" "	.08 "	41	42	....	.04 "	0	0
4 ..	" "	" "	....	....	.001 cc.	.06 "	8	10.4
5 ..	" "	.04 "	21	21	....	.04 "	20	21
6 ..	" "	" "	....	....	.001 cc.	.06 "	32.5	31.2
7 ..	" "	" "	....	....	Water ad.	2 "	32	31.2
8 ..	" "	.02 "	12	10.4	....	.06 "	26.5	31.2
9 ..	" "	" "	....	....	....	.04 "	19	20.8
10 ..	" "	" "	....	....	.001 cc.	.06 "	36.5	31.2
11 ..	" "	" "	....	....	Water ad.	2 "	40	40
12 ..	.001 cc.	.06 "	22.5	21	....	.04 cc.	0	0
13 ..	" "	" "	....	....	.001 cc.	.06 "	20.5	20.5
14 ..	" "	" "	....	....	Water ad.	2 "	30	31.2
15 ..	" "	.04 "	21	21	....	.04 "	0	0
16 ..	" "	" "	....	....	.001 cc.	.06 "	20.5	20.8
17 ..	" "	.02 "	11.5	10.5	....	.04 "	9	10.4
18 ..	" "	" "	....	....	.001 cc.	.06 "	30.5	31.2
19 ..	" "	" "	....	....	Water ad.	2 "	40	41.6
20 ..	....	.1 "	6	(5 + 2.5).	Water ad.	2 "	45	44.5
21 ..	....	.08 "	5	(4 + 2).				
22 ..	....	.04 "	3	(2 + 1).				
23 ..	....	.02 "	1.5	(1 + .5).				
24 ..	....	....	B, 1 cc.; water, 1 cc.	52				
25 ..	....	....	B, 5 cc.; water, 1.5 cc.	26				
26 ..	....	.1 cc.	Saline ad., 2 cc.	2.5				
27 ..	....	....	B1 Saline ad., 2 cc.	0.				

There was a certain amount of color in the complement added as follows:

.1	cc.	reads	2.5
.08	"	"	2
.06	"	"	1.5
.05	"	"	1
.02	"	"	.5

This is subtracted from the observed Fleischl readings and gives the true readings. Complement alone also causes some hemolysis (Nos. 20 to 23). If .02 cubic centimeter C produces 1.5 scale, .5 of this is due to the color of the C itself, and the series should be for .02, .04, .08 and .1 cubic centimeter C 1.5, 3, 6, and 7.5. That this is more nearly correct and that the error is due to reading is shown by the fact that the complete hemolysis of the washed remaining corpuscles gives a reading of 45, which is equal to 52, less the theoretical hemolysis by .1 cubic centimeter C alone or 7.5.

The close correspondence of the observed and calculated figures with the exception of Nos. 8 and 10 is certainly most marked. The hemolytic power of the serum is ca. .0025 (.00248), that amount completely hemolyzing one cubic centimeter of "Standard" suspension, containing 1×10^9 erythrocytes.

EXPERIMENT XI. 10/7/07.

No.	A3.	C5.	B8.	Saline.	Fleischl.	Calculated.	No. rbc. remaining.
1....	.002 cc.	.1 cc.	1 cc.	1 cc.	30 + +	32	
2....	" "	.08 "	" "	" "	30 + +	32	
3....	.001 "	.06 "	" "	" "	15	16	
4....	" "	.04 "	" "	" "	15	16	$.30 \times 10^6$ per cm.
5....	.0006 "	.03 "	" "	" "	9 +	9.6	
6....	" "	.024 "	" "	" "	9 +	9.6	$.40 \times 10^6$ per cm.

B8. Hemoglobin = 80%.
Erythrocytes = 1×10^6 per cm.
∴ Hemoglobin = .8 g. in 100 cc.

$$\frac{8 \times 10^{-3} \text{ g. in 1 cc.}}{1 \times 10^{9} \text{ rbc. in 1 cc.}} = \left\{ \begin{array}{l} 8 \times 10^{-12} \text{ g. in} \\ \text{one erythro-} \\ \text{cyte.} \end{array} \right.$$

In tube No. 4 a count of the deposited corpuscles gives $.3 \times 10^6$ per cm. or $.6 \times 10^9$ in the 2 cc. 16/40 of the hemoglobin is dissolved or 40%, leaving 60% undissolved, or 4.8 g. $\times 10^{-3}$ in the tube (2 cc.). Thus the corpuscular content of hemoglobin is again estimated as follows:

$$\frac{4.8 \times 10^{-3}}{.6 \times 10^{9}} = 8 \times 10^{-12} \text{ g. in one erythrocyte.}$$

From this experiment it would seem to be a fact that the corpuscles remaining in a partially hemolyzed mixture contain the same quantity hemoglobin as those of the original suspension. The previous experiment, XXXIII., showed also that when such corpuscles, to which have been added enough complement to completely saturate the amboceptor present, are thoroughly washed and a further amount of complement added, no more hemolysis takes place. In other words, each corpuscle at once takes up the maximum amount of amboceptor which it is capable of absorbing, and on the addition of complement is hemolyzed completely, no partial hemolysis of the corpuscle taking place.

From the foregoing experiments we concluded that it may be possible to obtain the definite quantitative relations of the three members of the hemolytic series and the resulting hemolysis.

The blood suspension employed should be of known corpuscular content: the most convenient contains one million erythrocytes per cubic millimeter. This may be varied, however, it only being necessary to know the exact number of red blood cells in suspension, and all estimations for comparison, being calculated from this number to correspond to a standard suspension, say of one million to the cubic millimeter. To this blood suspension is added the desired quantities of amboceptor and complement. The former, obtained as described above, was found to be constant in combining relations (except in a few cases in which the variation may be explained). This serum was diluted 1/100 or 1/500 with saline solution and added to the blood in required amounts. The fresh complement was also diluted 1/10. The total quantity in each tube was made up to two cubic centimeters by the addition of one per cent sodium chloride solution and the whole placed in the incubator at 37° C. for one hour, and afterward in the cold for two or three hours. In some cases the tubes remained in the ice-chest over night, the clear colored liquid being tested for the amount of hemolysis the next morning; in others, they were immediately centrifugalized and the hemoglobin dissolved estimated. There seemed to be no difference in the results in the two methods.

Using these methods and with care in measurements of quantities, sufficiently accurate results have been obtained, we think, to warrant the view that there is, as we stated, a definite numerical relationship between the amounts of immune body, complement, red blood cell, and resultant hemolysis.

In the various experiments, the hemoglobin content of the corpuscle varies considerably in the different sheep bleedings and even in the different suspensions from the same bleeding, the same quantity of amboceptor is anchored to each blood cell, *viz.:* 2.5×10^{-12} cubic centimeters; as most markedly shown in the latter experiments, in the earlier ones more errors of technic having crept in.

In each separate experiment, the quantity of amboceptor used is in direct proportion to the percentage of hemolysis. In different suspensions the hemoglobin content varies and, consequently, the amboceptor dissolved is not in proportion to the actual quantity of hemoglobin, but to the percentage to the total amount present.

The number of erythrocytes hemolyzed is in direct ratio to the quantity of amboceptor.

The hemoglobin content of red cells remaining in a partially hemolyzed blood is the same as those of the original suspension.

Serum obtained from eight immunized rabbits was constant in amboceptor content. (The only exception was two sera obtained as second and third bleedings from a rabbit that had undergone a long series of injections.)

The relation of fresh guinea-pig's serum used as complement and amboceptor is absolutely constant, these substances uniting in the proportion of forty to one.

Fresh guinea-pig's serum is constant in its complement content.

In fine, we find that .0025 cubic centimeter of hemolytic rabbit's serum immunized by the injection of sheep's corpuscles, in combination with forty times that amount of fresh guinea-pig's serum as complement, will completely hemolyze one billion blood corpuscles of the sheep. All of the blood suspensions employed were of the blood of one sheep. It might be possible that there would be a variation in these relations in different sheep.

We believe that such determinations as described must be made before definite conclusions may be drawn from experiments in which the phenomenon of hemolysis is used as an indicator in specific reactions of blood sera.

AN ACUTE PANCREATITIS WITHOUT NECROSIS OR BACTERIAL INVASION.*

CHANNING FROTHINGHAM, JR., M.D.

Christian and Germain[1] in 1904 reported a case of early acute pancreatitis, presumably of bacterial origin, without hemorrhage. Since that time several cases have come to autopsy in the pathological laboratory of the Boston City Hospital which showed an increase of polynuclear leucocytes in the pancreas. These cases did not present any gross pathological conditions, and it was only during the customary histological examination that the condition was noted. Is the invasion of these polynuclear leucocytes due to the presence of bacteria or to that of some other agent which attracts them?

The lesion in the pancreas in these cases varies considerably in extent. The character of the lesion seems to be the same, however, whether it occurs diffusely throughout the organ, in large local areas or in a few small foci. The islands of Langerhans are spared throughout even in the most severe cases. The lesion consists in an increase of the polynuclear leucocytes which have migrated from the vessels (see Figs. 1 and 2). These leucocytes work their way into the lumina of the glands between the epithelial cells. In the mild cases the epithelium of the gland does not present any abnormal appearance. In the severe cases, however, the cells present degenerative changes. The cytoplasm of the cells stains poorly and is diminished in amount. The nuclei have lost a considerable part of their chromatin network and the nucleoli often take a purplish stain. The glandular epithelium is distorted in shape and some of the cells have desquamated or have been crowded into the lumen by the invading leucocytes. In some cases the lumen is dilated and crowded with

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* From the laboratory of the Department of the Theory and Practice of Physic, Harvard University. Published with the approval of the Committee, as part of the work done under a Bullard Fellowship, 1908–1909. Received for publication Feb. 3, 1909.

polynuclear leucocytes and desquamated epithelial cells (see Fig. 3).

The smaller ducts, and in the severer cases the largest, show polynuclear leucocytes working towards the lumen between the epithelial cells (see Fig. 4). In no case were polynuclear leucocytes seen migrating into the islands of Langerhans. In no place was there a definite necrosis of the epithelium, but only this above described degeneration. Bacteria and other agents which might attract the polynuclear leucocytes were not found in any of the cases.

The clinical records of eight of the more marked cases were investigated in order to see if there were any symptoms which accompanied this condition. Of the eight three died of pneumonia, two of scarlet fever, one of erysipelas, one of ulcerative colitis, and one of general peritonitis. In none of these cases were there any symptoms referable to the pancreas. Since now neither the clinical nor the pathological signs suggested an acute pancreatitis of bacterial origin it seems fair to assume that the presence of these polynuclear leucocytes must be due to some other cause.

The possibility of this condition of the pancreas being allied with some special disease or set of diseases was next considered. For this purpose seventeen cases were taken which showed the above described condition in marked or moderate degree. Among these were one of diphtheria, one of scarlet fever, six of pneumonia, one of acute endocarditis, one of acute pericarditis, two of meningitis, one of erysipelas, one of ulcerative colitis, one of general peritonitis, one of general sepsis following scarlet fever, and one of chronic nephritis. The seventeen cases showed at least a moderate degree of leucocyte invasion. Several others showed a very slight, either local or diffuse, invasion of polynuclear leucocytes. Of these three were acute infections, one pericarditis, two pneumonia; and two were chronic diseases, one carcinoma, one tuberculosis of lungs and spleen with subcutaneous hemorrhages and persistent thymus. Of course many similar cases both acute and chronic occur which do not present lesions of the pancreas.

The series shows that the condition in the pancreas is not referable to any one or any set of diseases and that it can occur in either acute or chronic disease, but more frequently in acute infectious diseases.

As definite facts are lacking as to the cause of the attraction of the polynuclear leucocytes into the lumina of the glands and ducts of the pancreas, the following theory is suggested. The secretion of the pancreas becomes changed so that in some way it has a chemotactic effect upon the polynuclear leucocytes. If this is true it must be the secretion after it has been secreted by the cells, for the polynuclear leucocytes in no instance are actually penetrating the cells. In other words, the parenchyma is not necrotic. The agents which so affect the pancreas cells as to alter their secretion occur in a wide variety of acute and chronic disease, but not invariably in these diseases. What these agents are or what lesions they produce in the cells to cause this change in the secretion is not shown by this set of cases. The fact that the islands of Langerhans are not affected adds strength to this theory.

In order to see if a similar condition occurred in other organs the liver in eight of the more pronounced cases was studied. In all eight a similar condition was found in regard to the smaller bile ducts and in some instances the bile capillaries. In some instances it was quite marked, in some, slight. The lesion consists of an increase of polynuclear leucocytes in the periportal connective tissue. These leucocytes are clustered around the bile ducts and in some instances are extending in between the epithelial cells lining them. There is no sign of necrosis or bacterial invasion. In the more marked cases the polynuclear leucocytes are extending into the periphery of the lobules themselves, and are seen working their way in between the parenchymal cells, apparently going towards the bile capillaries which run within the liver trabeculæ. Here again, apparently, the bile had been so changed as to call forth the leucocytes. In the liver the smaller ducts seem to be most involved, while in the pancreas the acini are usually more involved than the ducts.

As these more pronounced pancreas lesions suggested that on healing they would show an increase of connective tissue with some loss of glandular structure, it was thought that by studying the past histories of some cases that showed chronic lesions in the pancreas some light might be thrown on the relation of this condition to other diseases. If these cases with chronic lesion had in the past suffered from a severe illness, it might suggest that this condition arose in severe infections which recover, as well as in fatal cases.

The past histories of thirteen cases which showed a chronic pancreatitis were examined, but the notes were so meager that no conclusion could be drawn in either direction.

The following conclusions seem justifiable:

(1.) Under certain conditions as yet undetermined, possibly just ante-mortem, the pancreatic secretion and the bile become so changed that they attract polynuclear leucocytes in such numbers as to cause considerable and occasionally extensive lesions.

(2.) This condition occurs more frequently in acute infectious diseases than in chronic diseases, though it may occur in either.

(3.) These lesions cause no clinical symptoms which are demonstrable.

[I am indebted to Dr. F. B. Mallory for valuable suggestions and to Mr. L. S. Brown for the photomicrographs.]

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EXPLANATION OF PLATE V.

FIG. 1. — Pancreas, x 187½. On one side normal pancreas. On the other parenchymal cells somewhat degenerated and marked invasion by polynuclear leucocytes.

FIG. 2. — Pancreas, x 187½. Foci of pancreas tissue showing the invasion with polynuclear leucocytes and degeneration of the parenchyma.

FIG. 3. — Pancreas, x 1,000. Dilated gland crypt filled with polynuclear leucocytes and desquamated epithelial cells. Parenchymal cells distorted and degenerated.

FIG. 4. — Pancreas, x 1,000. Epithelium of large duct. Polynuclear leucocytes wandering in between epithelial cells. (Arrows point to the leucocytes.)

SOME STUDIES OF THE PHYSIOLOGICAL LEUCOCYTE CONTENT OF COW'S MILK.*

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In coming to any satisfactory conclusions on the vexed question of the significance of leucocytes in milk and the determination of a sanitary standard for use by health authorities, it is essential that we know as much as possible regarding the physiological leucocyte content of cow's milk.

The leucocyte is a wandering cell passing readily through the walls of the blood vessels in the performance of its function of a phagocyte, and its presence is to be expected to some extent in every tissue or secretion of the body, but only as a result of injury causing inflammation do these cells collect in large numbers in any one locality aside from their natural stronghold, the lymphatic tissue. Let such an injury take place, and the leucocytes immediately swarm from the adjacent tissues and through the blood stream to the focus of the injury, passing, by their own ameboid motion, through the interstices of the tissues and the walls of the vessels and proceed to perform their function of removing the effete material or dangerous bacteria.

Entirely independent of any such rapid local accumulation due thus to accidental external causes, we find in the blood stream itself a number of leucocytes, varying considerably as a result of not only pathological causes, local and general, but also coincident with certain physiological processes — such as digestion, violent exercise, parturition.

In milk, as in the blood stream, we find normally a varying number of leucocytes, although in the milk they have undergone changes which render them indistinguishable from pus cells. Until some method is devised for differentiating leucocytes from pus cells in milk, it is necessary to fix a limit to the number allowable in wholesome milk.

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* Paper read before the laboratory section of the American Public Health Association at Winnipeg, Manitoba, August, 1908. Received for publication Feb. 9, 1907.

Various methods of enumerating leucocytes have been used by students of the subject, each establishing an arbitrary standard based upon a method of counting. Stokes and Wegefarth[1] were the first to establish a standard based upon the following method of enumeration. A definite quantity of milk was centrifugalized, the sediment spread over a definite area, stained and examined with a one-twelfth-inch lens. They considered that five cells to the field of the microscope indicated pus. Bergy[2] regarded ten cells per field as the limit. Stewart[3] adopted twenty-three cells per field as a standard. Slack[4] considered fifty cells per field the maximum. The above investigators used different modifications of Stokes' method. Doane-Buckley[5] employed the following method: Ten cubic centimeters of milk were centrifugalized, the cream removed, the milk drawn off nearly down to the sediment, two drops of methylene blue added and then water to make up to one cubic centimeter. This was thoroughly mixed and a Thoma-Ziess blood counter used for enumerating the cells. They considered five hundred thousand leucocytes per cubic centimeter as an indication of pus, and expressed doubt as to whether this was high enough. Russell and Hoffman[6] found that cows without the slightest indication or history of udder trouble frequently had leucocyte counts as high as one million or even higher.

These and other investigators have shown that there is a wide variation in the leucocyte content of milk even when taken from the same cow day by day. As yet no entirely satisfactory conclusions as to the cause of this variation have been made, although certain facts relating to the secretion of milk explain some of them.

Thinking it possible that fluctuation in the leucocytes of the blood stream might be reflected in the milk, we made some parallel leucocyte counts of blood and milk during the summer of 1907. The cows selected were those in a herd of the Vermont Experiment Station, by consequence animals whose histories were known and subject to careful feeding and veterinary inspection. Some of these cows had histories of abortion within a short time and a number of them had had garget. None were perceptibly suffering from acute symptoms of these conditions at the time of examination. The milk and blood (from the ear) were taken on the morning in each case. The Doane-Buckley method, without a stain, was used for making the milk counts and the usual Thoma-Zeiss method for the blood. After completing the first series the cows, having high leucocyte counts in either blood, or milk — five hundred thousand in one case, and ten thousand in the other — or both, were subjected to two subsequent examinations, after thirty-three and forty-two days. The tabulated results of these counts (Table I.) show that in fifty-eight cows examined there were twelve, or more than twenty per cent, which showed a milk leucocyte count of over five hundred thousand, five (eight and one-half per cent) with a milk count of over one million; that there were seven (twelve per cent) with a blood leucocyte count of over ten thousand per cubic millimeter; one (two per cent) with a blood count of over twenty thousand; and twenty (thirty-four per cent) with a low blood count under six thousand; that there were none having both high blood and milk count and that the number with low blood count and a high milk count was six (ten per cent of the whole number and fifty per cent of the number showing high milk count).

Thus we see (1) that the average number of leucocytes of the cow's blood is between six and ten thousand per cubic millimeter subject to no such variations as the milk leucocyte content; (2) that there is absolutely no relation between the general blood condition and the leucocyte content of the milk — thus indicating that variations in the latter are due to local causes entirely; (3) that there is apparently a tendency in certain cows to a high leucocyte content in milk over a considerable time.

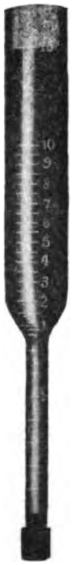
Realizing that this lot of tests was hardly a fair one for determining the physiological leucocyte content in the milk of perfectly normal cows, as many of this series had bad histories, we determined to choose a number of healthy animals kept under careful inspection and make daily tests of the milk, through the entire period of lactation. Accordingly, two healthy grade Jerseys, Doris and Tansy, aged respectively seven and five years, were selected from the Experiment Station herd loaned to us for this purpose through the kindness of Profs. J. L. Hills and C. L. Beach and milk taken from them night and morning from the time of dropping their calves until they were dried off. During all this time both of these animals were kept under exactly similar conditions on regular feed with the exception of a change (on May twenty-third) from dry feed to grass. They were carefully watched and subjected to frequent veterinary inspection and during the entire period covered by the accompanying tables were pronounced to be in perfect health.

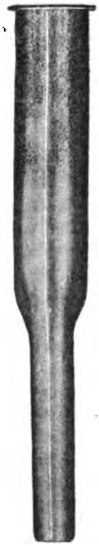
An analysis of the counts as submitted in the accompanying tables (II. and III.) shows that there was no material difference in the leucocyte content of the milk of the two cows, so that the results can be considered together. One and one-fifth per cent of the entire one thousand one hundred and sixty-seven samples showed leucocytes below ten thousand per cubic centimeter; seven per cent between ten and twenty thousand; sixty-one per cent between twenty and one hundred thousand; twenty-nine per cent between one hundred and five hundred thousand; and only one per cent above five hundred thousand. The most surprising fact noticeable in our work is the remarkably sudden fluctuations which occurred occasionally with no apparent cause. Thus, on July fifteenth Tansy showed a count of ten thousand and seventy-five thousand, rising on the sixteenth to five hundred and thirty-five thousand and one million four hundred and forty thousand, dropping again in two days to ninety thousand. Doris on the same day showed no increase. Again on the evening of July twenty-seventh, Doris' milk contained seventy-five thousand leucocytes, suddenly increasing to one hundred and thirty-four and nine hundred and fifty thousand in the next two milkings, dropping again to ninety thousand on the second day after. During this time the other cow showed no increase, registering sixty thousand on the same day of Doris' maximum.

We feel that these sudden great fluctuations must be due to some real cause, so trivial and transient as to be overlooked by our necessarily gross methods of examination, but possibly demonstrable by more careful examination than we were able to make.

The results of this work have tended to shake our confidence in an arbitrary numerical leucocyte standard as a reliable criterion of the sanitary fitness of milk when taken from single cows, yet we do believe that the physiological average is considerably below five hundred thousand — only one per cent of the counts made by us were above this number — and that very seldom would the count of the mixed milk of ten or more cows go above this figure unless there were present abnormal conditions in some of the animals. With the two cows selected by us, the average was above five hundred thousand only three times in a total of five hundred and thirty-eight. It is fair to presume that the additional introduction of one or more normal cows into the series would have materially lowered the percentage as there seems to be no relation between the high counts in the milk of the two cows when kept on regular diet.

We have devised a tube for quantitative and qualitative analyses of milk sediments.





This tube has many advantages over the ordinary centrifuge tube. First, it measures the sediment more accurately. Second, smears of the sediment can be made in the same manner as with the Slack tube. Third, the column of sediment tends to become stratified so that the different elements can be easily made out. Fourth, the tubes are much more easily cleaned. With this tube we have been able to detect without microscopical examination the presence of cases of acute mastitis when the milk was diluted thirty times with normal milk. For ordinary routine work we think that centrifugalization in these tubes, using one-tenth cubic centimeter of a leucocyte sediment to fifteen cubic centimeters of milk, in a filled tube, as a maximum limit, will be of as much value as a count. The character as well as the quantity of the sediment should of course be taken into consideration. A sediment from cases of even slight mastitis practically always have a yellowish or pinkish tinge of pus, mixed with a smaller or larger amount of blood. Microscopical examination usually shows red cells as well as pus cells. This test should of course be supplemented by an actual examination of the herds in every case.

STUDIES IN NORMAL LEUCOCYTE CONTENT OF COWS' MILK.

TABLE I.

Number of leucocytes per cubic centimeter in blood (from ear) and milk taken at the same time (000 omitted).

Cow.	1st Count, June 29.		2d Count, July 30.		3d Count, September 11.	
	Blood.	Milk.	Blood.	Milk.	Blood.	Milk.
1.....	6,500	83				
2.....	6,300	41				
3.....	5,600	13				
4.....	6,800	350				
5.....	5,400	23				
6.....	5,600	78				
7.....	7,160	115				

TABLE I. — *Continued.*

Cow.	1st Count, June 29.		2d Count, July 30.		3d Count, September 11.	
	Blood.	Milk.	Blood.	Milk.	Blood.	Milk.
8.....	8,700	171				
9.....	5,000	429				
10.....	5,800	43				
11.....	5,800	72				
12.....	3,400	46				
13.....	8,000	52				
14.....	8,200	86	8,000	109		
15.....	11,400	130	7,400	85	102,000	265
16.....	5,800	221				
17.....	8,200	385				
18.....	5,200	415				
19.....	5,000	19				
20.....	10,400	36	12,800	30	11,000	44
21.....	....	24				
22.....	....	134				
23.....	6,600	1,470	8,600	595	6,500	1,065
24.....	6,000	271	....	....	6,400	930
25.....	5,500	1,185	5,800	875	6,600	710
26.....	9,600	272				
27.....	8,600	411				
28.....	6,000	8,800	1,200	57	8,200	144
29.....	5,000	524	5,800	935	7,600	835
30.....	8,600	510	6,400	560	6,000	555
31.....	12,800	56	8,700	80	9,600	147
32.....	7,000	92				
33.....	11,000	315	11,130	24	16,400	79
34.....	6,200	380	10,600	846	13,000	775

TABLE I. — *Concluded.*

Cow.	1st Count, June 29.		2d Count, July 30.		3d Count, September 11.	
	Blood.	Milk.	Blood.	Milk.	Blood.	Milk.
35.....	7,400	500	7,000	640	6,000	530
36.....	5,600	655	7,400	890	7,000	530
37.....	8,000	124	....	....	7,000	45
38.....	12,600	225	11,400	33	9,800	34
39.....	20,000	24	11,200	90	10,000	110
40.....	5,200	691	6,600	48	7,800	475
41.....	8,400	45				
42.....	5,600	81				
43.....	5,200	250				
44.....	7,400	300				
45.....	5,600	167				
46.....	8,000	106				
47.....	9,200	225				
48.....	10,400	25				
49.....	6,000	255				
50.....	7,600	295				
51.....	6,000	15				
52.....	8,800	10				
53.....	4,800	745	2,400	580	6,400	1,105
54.....	8,400	85				
55.....	11,200	165	10,000	930	10,400	345
56.....	5,600	640	....	....	5,620	107
57.....	8,000	105				

Table showing the results of one thousand one hundred and sixty-seven leucocyte counts made from the night and morning milk of two cows, Doris (24 of Table I.) and Tansy (4 of Table I.), from the beginning of lactation:

TABLE II. — *Doris.*

Number of Counts between the Numbers (Inclusive of Lower Number).					Total.
10,000 and 20,000.	20,000 and 100,000.	100,000 and 500,000.	500,000 and 1,000,000.	1,000,000 and 5,000,000.	
9	316	211	8*	2†	546

Highest count, 2,110,000 in first two days of lactation.
Lowest count, 10,000.
Average of all counts, 112,203.
* Two of these in first two, two in last two days of lactation.
† In first two days of lactation.

TABLE III. — *Tansy.*

Number of Counts between the Numbers (Inclusive of Lower Number).						Total.
Below 10,000.	10,000 and 20,000.	20,000 and 100,000.	100,000 and 500,000.	500,000 and 1,000,000.	1,000,000 and 5,000,000.	
14	73	394	131	7	2	621

Highest count, 1,444,000.
Lowest count, 6,000.
Average of all counts, 84,341.

$1^1/_5$% of total number of counts below 10,000.
7% " " " " " between 10–20,000.
61% " " " " " " 20–100,000.
29% " " " " " " 100–500,000.
1% " " " " " above 500,000.
Only 3 of 538 averages of milk of two cows above 500,000.

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A REPORT OF ELEVEN CASES OF STAPHYLOCOCCUS INFECTION TREATED WITH LEUCOCYTE EXTRACT.*

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In continuing their work upon the therapeutic value of leucocyte extracts,[1] the writers have had occasion to make observations upon the curative influence of these extracts upon cases of staphylococcus infections in man, which are briefly given below. In all cases the leucocytes were extracted in distilled water as in the previous work, and were administered to the patients by subcutaneous injections. The animals used for obtaining the leucocytes were rabbits.

Cases of localized staphylococcus infection (furunculosis) treated with leucocyte extract:

Case 1. — Mr. D. M., twenty-five years old (patient of Dr. A. V. S. Lambert), strong, healthy, polo player, has been living active outdoor life for the last four months. For about six weeks has been troubled with boils located chiefly in the right axilla. Staphylococcus aureus cultivated from boils. These have come out in groups of two or three at intervals of about a week. During the past five weeks has always had one or two actively inflamed. They have varied in size from small pimples to boils having a base of two to four centimeters. For the past two weeks has been under surgical treatment with incision and surgical antisepsis.

May 8, 1908. Ten cubic centimeters of leucocyte extract injected.

May 12. Ten cubic centimeters of leucocyte extract injected.

May 14. Ten cubic centimeters of leucocyte extract injected.

After the first injection of leucocyte extract no new furuncles appeared, and those already existing began to subside. Seen one week after last injection; at that time found to be rapidly clearing up.

Communication from Dr. Lambert six months after last injection states that after first leucocyte extract injection no boils appeared and patient seemed cured.

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* Received for publication Feb. 24, 1909.

Case 2. — G. P., assistant carpenter St. Luke's Hospital, twenty-two years old, Swede, unusually healthy young man, athlete, general health excellent. For three years has been troubled with persistent pimples on the face. For the past four months has had boils on the neck and behind the ears, a new one developing every other day. For the last few weeks boils have appeared on both legs and in the left axilla. For the past two weeks has never gone two days without the development of a new furuncle. These have been growing steadily more severe and during the past week he has developed a very painful furuncle with extensive induration within the external meatus of the right ear.

On examination he was found to have at least fifteen to twenty boils on the back of the neck and behind the ears. Staphylococcus aureus was cultivated from boils.

October 7. Ten cubic centimeters of leucocyte extract injected.

October 8. No new boils appearing, and old ones improved.

October 10. Ten cubic centimeters leucocyte extract. No new boils appear.

October 11. Ten cubic centimeters given prophylactically.

No new development of furuncles until November 28, when a small pimple appeared on the back of the neck, which was quite angry and painful, and ten cubic centimeters of leucocyte extract was injected.

December 1. No new boils have developed. Patient appears entirely well.

Case 3. — Mr. A. von der M., twenty-nine years old, broker, healthy young man, has just returned from long summer vacation during which he has lived an open air life. General health excellent. During the past four months has had constant trouble with boils which developed on the right leg. They have always been large, measuring from five to eight centimeters in diameter, thickly indurated, and extremely painful. Altogether has had about thirty during this time, and when seen on October 14, has two, which are healing, on the right buttock and a very large and active one at the base of the large toe on the right foot, extending over the dorsum of the foot about five centimeters. Discharging; Staphylococcus pyogenes aureus isolated from discharge.

October 14. Ten cubic centimeters of leucocyte extract injected, and boil dressed.

October 17. Ten cubic centimeters of leucocyte extract injected. The boil on the foot rapidly healing at this time; discharge diminished, and induration softening. After last injection no new boils appeared.

Heard from two months after last injection; no new boils.

Case 4. — Dr. M., house physician at a New York hospital. For the past seven years has suffered from periodical attacks of boils appearing at intervals of about three weeks, usually about fifteen to twenty-five at a time; often very large, the largest having an indurated base of almost one inch in diameter. He has never been entirely free at any time, his complexion being always very bad; face and back covered with furuncles at various stages of development. Has been systematically treated by various physicians; never with much relief. Seen on Oct. 27, 1908; had about thirty boils on back and above the waist line, varying in size, the largest about 1.5 centimeters in diameter.

October 27. Ten cubic centimeters of leucocyte extract injected.

October 29. Ten cubic centimeters of leucocyte extract injected. Following this injection there was some distinct improvement in the boils, which at the time when treatment was begun, however, were in the stage of retrogression.

November 13. It seemed to patient that one or two small new furuncles were appearing in the manner in which periodical attacks usually began. Ten cubic centimeters of leucocyte extract were injected. The patient's condition showed distinct improvement; only a few boils appeared. Back shows unmistakable signs of clearing up; face likewise, but less markedly.

December 2. Ten cubic centimeters of leucocyte extract injected. Patient continues to improve. Boils appear more rarely, are smaller, and show a tendency to become more acute and of shorter duration.

January 1. Patient withdrawn from observation; markedly improved, though not absolutely cured.

Case 5. — Mr. J. R. S., patient of Dr. Joseph A. Blake. Large boil on back of neck, indurated, about two to two and one-half centimeters in diameter, extremely sore.

October 28. Ten cubic centimeters of leucocyte extract injected, 6 P.M.

By the following day the boil appeared to be drying up. The pain had disappeared. and there was rapid retrogression. Owing to the fact that only one boil appeared in this case little conclusion can be drawn from it.

Case 6. — Mr. M., lawyer, twenty-five years old; excellent general condition. Since 1907 has been troubled with boils which have appeared in the axilla. During the winter of 1907–1908 had four boils on the back of the neck lasting about two weeks each. From May to June, 1908, had a furuncle on the neck which was followed by others in rapid succession. During June was free for about three weeks. From June 24 to September 12, another attack, boils appearing in quick succession in the axilla and on the back of the neck. Following this a few isolated boils appeared in various places which kept him more or less uncomfortable until November, 1909. First seen November 18. Patient has healing boil on the back of the neck.

November 18. Ten cubic centimeters of leucocyte extract injected.

November 24. Ten cubic centimeters of leucocyte extract injected.

November 26. Ten cubic centimeters of leucocyte extract injected.

Patient reports that he feels very much better; appetite and general condition satisfactory. No new boils appear. Patient apparently cured of boils when seen January 14, two months after first injection.

Case 7. — Mr. DeN., twenty-nine years old, broker. General health excellent; has just returned from prolonged summer vacation. Since Oct. 15, 1908, patient has had numerous boils which have appeared chiefly on the lower extremities and on the buttocks. Always extremely large, and very painful, and lasted several weeks each. When first seen (November 19) had a large boil on left buttock, with an indurated base of almost ten centimeters in diameter, which was discharging pus from which Staphylococcus pyogenes aureus was isolated.

November 19. Ten cubic centimeters of leucocyte extract injected.

November 23. Ten cubic centimeters of leucocyte extract injected.

November 25. Ten cubic centimeters of leucocyte extract injected.

There was rapid healing of the boil present at the time of the first injection, and when heard from six weeks later no new ones had developed. Case apparently cured.

Case 8. — Infant, born at the Sloane Maternity Hospital, about nine days old. Shortly after birth began to have furuncles on neck, two or three being present at the same time. During this time the child lost weight and appeared to be failing.

November 24. Ten cubic centimeters of leucocyte extract injected.

November 26. No new furuncles have developed.

November 28. Ten cubic centimeters of leucocyte extract injected. Child apparently cured.

Case 9. — Mr. E., healthy man; good general condition. For fourteen years has had boils on the back of the neck; has rarely been free for two months at a time. Patient of Dr. A. V. S. Lambert.

January 7. Ten cubic centimeters of leucocyte extract injected. At this time a boil was developing on the back of the neck.

January 8. Ten cubic centimeters of leucocyte extract injected. Boil on back of neck seems to be disappearing.

January 14. Ten cubic centimeters of leucocyte extract injected. Boil seems to be beginning on back of neck.

January 15. Ten cubic centimeters of leucocyte extract injected. No boils develop.

February 1. No new boils.

Although this case cannot be definitely assumed to be cured as yet, the patient having had intervals of two months at various periods during his illness, nevertheless the apparent retrogression of developing boils during the progress of an attack, and the freedom from all lesions for six weeks after injection, make it appear extremely likely that the leucocyte extract has been effectual.

Case 10. — Mrs. M., St. Luke's Hospital, patient of Dr. C. W. Cutler. Patient had been suffering from chronic rhinitis with ulcerations around the opening of the nasal duct. Six months before present illness she had an operation for the removal of the inferior turbinate bone. Three weeks before present illness, the patient developed an acute dacryocystitis. There were several attacks of this condition following the introduction of a probe. There appeared to be a stricture in the nasal duct. Staphylococcus pyogenes aureus was at this time isolated from the inflamed area in lachrymal duct and from the nose. Following an attempt to correct the stricture on Jan. 12, 1909, infection from the nose traveled up through the nasal duct into the orbit. The patient's temperature rose to 103.4° F. and a rapidly spreading acute inflammation developed in the orbit, from the pus of which Staphylococcus pyogenes aureus was recovered in pure culture. There was marked exophthalmos, complete immobility, and protrusion of the eyeball. It was the opinion of the attending physician that the tissues behind the eye were edematous and that the orbit probably contained pus.

January 13. Ten cubic centimeters of leucocyte extract injected. Temperature drops to 100.2° F. on following day, but rises again to 101.8° F. in afternoon. On January 14, two injections of ten cubic centimeters of leucocyte extract.

January 15. Temperature drops to 99.8°. Marked improvement in general condition and in local lesion. During the night of January 15, temperature again rose to 100.8°, but dropped on January 16, to 99° F. Ten cubic centimeters of leucocyte extract injected. Temperature does not go above 100.2° F., on the following morning being 99.4°. General improvement progresses, and there is marked clearing up of local lesions. Rapid and uneventful convalescence.

In this case there was a very acute staphylococcus infection in a region not amenable to surgical treatment and drainage, except by recourse to very radical measures. Following each injection of leucocyte extract there was a marked improvement. Both the rapid response in the patient's condition and temperature, and the clearing up of the local lesion, seem to indicate definitely that the leucocyte extract was effectual.

Case 11. — Mrs. B.; puerperium. Soon after birth of child developed pain in right breast, which was deeply situated and diagnosed by attending physician as a deep abscess of the breast. Deep massage of the breast was followed by a small amount of milk, which, however, was soon replaced by yellowish drops of pus. Microscopical examination of culture revealed Staphylococcus pyogenes aureus. Massage and other local treatment continued for several weeks. Condition improves, but when massage is omitted returns to original acuteness.

January 21. Ten cubic centimeters of leucocyte extract injected in the morning. Injection of same quantity repeated in afternoon. Following these two injections the pus seems to disappear and there is marked improvement in condition of breast which returns to normal.

Eleven cases of staphylococcus infection have been systematically treated and observed. In all but three of these the processes consisted of furunculosis of a chronic nature and had lasted, in spite of surgical and dietetic treatment and in some cases in spite of the most desirable hygienic conditions, for periods ranging from several months to four or five years. In none of the cases did the leucocyte extract fail to exert a markedly beneficial action, not only noticeable to the experimenters, but conceded by the attending physicians and by the patients themselves. In all but one of the more chronic cases there was apparently complete cure, the patients themselves coming back for treatment if, after weeks of freedom, a suspicious acne pustule attracted their attention. The few acute cases which have come under observation have been even more striking in the immediately apparent local and general improvement following the injections. This is most patently illustrated in Case 10 of the series described.

In addition to these eleven cases, two cases of chronic acne were treated. Both of these were examples of the most severe type of indurated acne, and had stubbornly resisted months of treatment by dermatologists. While the time of treatment given to these cases has been too short to warrant final judgment, there is no doubt in our minds that the lesions in both cases after four or five injections of leucocyte extracts have taken on a less indurated and more acute character — a fact which argues in favor of a higher local resistance. Another case of chronic frontal sinus infection with Staphylococcus pyogenes aureus has been favorably influenced by the leucocyte extract; but final judgment upon this case must be reserved until a longer period of observation has elapsed.

On the whole, the writers have no hesitation in concluding that marked improvement in, and often cure of, localized staphylococcus infection may be obtained by careful and systematic treatment with leucocyte extract.

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LOCALIZED ANAPHYLACTIC INTOXICATION IN CHILDREN FOLLOWING THE REPEATED INJECTION OF ANTITOXIN.*

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V. Pirquet and Schick[1] in their admirable monograph on the "serum sickness" have described the untoward effects of single and of repeated injections of horse serum (antitoxin) in human beings. The cutaneous irritative symptoms which occur six to ten days after a primary injection are known to be dependent on the individual animal that has furnished the serum rather than on any particularly hypersusceptible condition of the patient. Of far greater academic interest as well as of striking clinical importance is the "accelerated" or "immediate" (beschleunigte) reaction which these authors have described. This reaction occurs primarily in patients that have been "sensitized" by a previous serum injection which may have been given months before; it does, however, also occur in certain instances in cases which are given serum for the first time. This accelerated reaction is characterized by the appearance within a few hours, and frequently within a few minutes, of some or all of the following symptoms: malaise, rise of temperature, urticaria or erythema, edema, cyanosis, dyspnea, and at times sudden death.

The results of v. Pirquet and Schick have been confirmed and amplified in a considerable series of cases by Goodall and by Currie. Goodall[2] found that of ninety cases of diphtheria that received two injections of serum, nine showed an immediate reaction on second injection. The time between injections varied from thirty-five to three hundred and sixty-three days. Currie[3] found that the second injection produced some symptoms in as many as sixty-six per cent of one hundred and fifteen cases examined. This author further emphasized the necessity and importance of a time interval between injections.

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* Received for publication Feb. 25, 1909.

The work of the past three years by Otto, Rosenau and Anderson, Gay and Southard, Besredka, and others on serum anaphylaxis in the guinea-pig has thrown much light on the mechanism of the serum sickness in human beings. Little if any doubt remains as to the perfect analogy of the two phenomena. It will be recalled that guinea-pigs that have received a small dose of horse serum will after a proper incubation period react violently to a second dose. The symptoms are first irritative then paralytic, and death, which occurs regularly when proper dosage and incubation period are observed, is always due to cessation of respiration. The cessation is due to overstimulation of an irritable respiratory center and not to its paralysis (Gay and Southard[4]). The symptoms of dyspnea and cyanosis in the severer human cases follow in detail the symptoms of guinea-pig anaphylactic intoxication, and death, when it occurs in man, is likewise due to cessation of respiration. It has further been remarked by Gillette[5] and others that those patients which react to a *primary* injection of serum are almost invariably afflicted with asthma, which disease may reasonably be attributed to some abnormality in the respiratory center. The further analogy between serum anaphylaxis in man and in guinea-pigs in respect to the effect of frequent doses of serum and the importance of the length of incubation periods between doses, on the symptoms involved, will be later considered.

As we have seen, the immediate systemic reaction following a second dose of horse serum in human beings corresponds to the intoxication in guinea-pig anaphylaxis. There has been, however, a form of reaction to alien serum in animals of which the human analogue has not yet been described. Arthus[6] some years ago described the occurrence of local aseptic necroses in rabbits that had been given several subcutaneous injections of horse serum at week intervals. The animals showed no reaction at the point of inoculation after the first three injections of five cubic centimeters each of serum. The fourth injection gave a white infiltrated area, the fifth a still further induration which took five or six days to resorb, and the sixth injection gave rise to actual necrosis. The point of inoculation in the successive doses is not the same. In rabbits this local reaction is much more constant than a systemic reaction, which may, however, follow a second or third dose when given intravenously. This local reaction was also found by Arthus in guinea-pigs, rats, and doves. In guinea-pigs its occurrence bears no direct or necessary relation to the occurrence of generalized symptoms (Lewis[7]).

We have found that this localized lesion of anaphylactic intoxication (Arthus' phenomenon) occurs frequently in children that have received two or more doses of antitoxin at frequent intervals. The frequency of occurrence of this local lesion varies directly with the number of injections.

It is a routine practice in the Children's Hospital to give the patients one thousand five hundred units of antitoxin (about five cubic centimeters) on their admission and to repeat the dose every twenty-one days so long as the child remains in the hospital or in the convalescent home which is connected with the hospital. The local reaction which we have noticed has occurred as early as the second injection and in some cases not until the sixth injection. The reaction appears within a few hours as a general swelling or edema of the whole region surrounding the point of injection; there is general reddening with marked local calor; this area soon becomes quite glazed and shiny, there is extreme tenderness, and if immediate local treatment, consisting of absolute rest with application of either heat or cold, is not instituted, the process goes on to moderately general tissue necrosis. As the swelling subsides under treatment the region becomes quite markedly indurated and hard, the tenderness and induration often continuing for one or two months, especially immediately about the point of injection.

In the following table, comprising a review of one thousand consecutive cases, are summarized the immediate symptoms both local and general which followed the injection of antitoxin. The table is divided in accordance with the serial case numbers.

TABLE I.

Immediate reactions following injections of antitoxin in children.

Case No.	No. of Injection.	Generalized Symptoms.					Local.
		Temperature.	Malaise.	Edema.	Rash.	Dyspnea.	
1	1st	101.2	Moderate.				
2	1st	101					
3	1st	Slight.	....	....	Marked.		
4	2d	....	....	....	....	....	In back (necrosis).
5	2d	101					
6	2d	Slight.	....	....	....	....	In thigh.
7	2d	....	Marked.	....	Urticaria.		
8	2d	....	Marked.	....	....	....	In back.
9	2d	Moderate.	Arthralgia.				
10	2d	....	....	....	....	....	Arm, marked.
11	2d	....	....	....	....	....	Arm, moderate.
12	2d	Slight.	....	....	Slight.		
13	2d	....	Marked, vomited.				
14	2d	105	....	....	....	....	Arm, marked.
15	2d	....	....	....	....	....	Thigh.
16	2d	....	....	....	....	....	Thigh.
17	2d	103	....	....	Urticaria.	....	Moderate.
18	2d	....	....	....	....	....	Thigh, moderate.
19	2d	102.5	....	....	....	....	Back.

2 ...	2d	103	...	...	...	...	Thigh.
20...	2d	101	...	...	Moderate.		
21...	2d	...	...	...	Urticaria.		
22...	2d	...	...	...	...	...	Thigh.
23...	2d	103	...	...	...	...	Moderate.
24...	2d	101					
25...	2d	...	Marked, vomiting, very restless.	Marked, of face.	Urticaria.		
26...	2d	...	Very marked.				
27...	2d	102					
28...	2d	101	...	...	...	...	Marked.
4...	3d	Marked.	Marked.	...	Urticaria.	...	Back.
29...	3d	Moderate.	...	...	...	...	Moderate.
30...	3d	102.2	...	...	...	...	Right thigh, moderate.
15...	3d	...	...	...	...	...	Thigh.
17...	3d	...	...	...	Moderate.	...	Back.
19...	3d	101	...	...	...	...	Thigh, moderate.
31...	3d	...	...	...	...	...	Moderate.
2 ...	3d	103.6	Quite sick.				
32...	3d	Slight.	...	...	...	...	Thigh, moderate.
33...	3d	Moderate.	...	...	...	...	Thigh, moderate.
34...	3d	...	...	...	...	...	Moderate.
35...	3d	102.2	Irregular pulse.				
36...	3d	101	...	...	Moderate.		
37 ...	3d	Marked.	...	...	...	...	Arm, marked.
38...	3d	100	...	...	Moderate.		

TABLE I. — *Continued.*

Case No.	No. of Injection.	Generalized Symptoms.					Local.
		Temperature.	Malaise.	Edema.	Rash.	Dyspnea.	
4	4th	Moderate.			Moderate.		Back.
39	4th						Back, moderate.
40	4th	103	Irregular heart.				
17	4th				Urticaria.		Marked.
19	4th	101					Thigh, moderate.
32	4th						Moderate.
33	4th						Moderate.
41	4th	Slight.					Slight.
42	4th						Slight.
35	4th						Moderate.
37	4th						Marked.
24	4th						Moderate.
43	4th	Marked.					Moderate.
4	5th		Less than 4th.		Slight.		Quite marked.
44	5th	102.8	Quite upset.				Moderate.
39	5th						Moderate.
40	5th		Marked.				Necrosis.
45	5th			General, especially of face.			

17	5th						Marked.
19	5th	100.6					Moderate.
2	5th	103	Quite sick.				Thigh, very marked.
33	5th	103.4					Thigh, very marked.
41	5th						Thigh, moderate.
42	5th						Back, moderate.
43	5th						Marked.
4	6th						Arm, very marked.
44	6th	Moderate.			Moderate.		Arm, very sore; glands in axilla.
40	6th						Arm, very sore.
45	6th			General, marked.			Thigh, marked.
46	6th	Moderate.					Moderate.
2	6th	102.4	Slight.				Very marked.
41	6th						Moderate.
42	6th						Thigh, moderate.
47	6th					Marked.	Arm, moderate.
48	6th					Moderate.	
49	6th		Irregular heart.		Moderate.	Moderate.	Slight.

To summarize the findings in this table:

Following a primary injection in one thousand cases only three (.03 per cent) gave any immediate reaction. The reactions in these three cases were general but very slight and confined principally to slight rises in temperature. They may scarcely be regarded as instances of the same reaction that occurs on reinjection.

Following a second injection in two hundred and eighty-one cases, twenty-six reacted (8.8 per cent). Of these twenty-six, ten gave only generalized symptoms, seven both generalized and local and nine local symptoms only.

Following a third injection in one hundred and three cases fifteen reacted (14.5 per cent). Of these fifteen two showed only generalized symptoms, nine both generalized and local, and four local symptoms alone.

Following a fourth injection in thirty-six cases, thirteen reacted (27.75 per cent). Of these thirteen two showed generalized symptoms alone, three both general and local, and eight localized only.

Following a fifth injection in twenty-five cases, twelve reacted (42.9 per cent). Of these twelve one only was with generalized symptoms, five with generalized and local symptoms, and six with pure local symptoms.

Following a sixth injection in fifteen cases, eleven reacted (seventy-four per cent). Of these eleven only one showed pure generalized symptoms, four both general and local, and six purely local.*

It is evident, then, that the occurrence of immediate reactions on reinjections with antitoxin varies directly with the number of injections given. Our local reaction of intoxication, moreover, increases gradually over the systemic or generalized reaction of v. Pirquet and Schick in proportion to the number of injections given at three-week intervals.

This latter fact, moreover, corresponds perfectly with the conditions in animals. In Arthus' experiments rabbits were given relatively large doses of serum at short intervals and showed the local reaction invariably but the general reaction seldom. In guinea-pigs the successful demonstration of the generalized reaction depends on a small initial dose and a relatively long incubation period; the period of incubation varies, indeed, directly with the number of moderate doses or the size of a single initial dose (Gay and Southard[8]). In guinea-pigs, moreover, the local reaction is unusual. V. Pirquet and Schick, Goodall, and Currie have dealt rather with a second injection following a primary sensitization at varying and usually at long intervals, and the reactions they have met with have been of necessity the systemic rather than the local. We have dealt on the other hand with cases given several injections at relatively short intervals (twenty-one days) which approximates the conditions noted by Arthus in his rabbits. It is quite probable that the mechanism of this form of local anaphylactic intoxication differs from the one that underlies the more systemic reactions.

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* It may be noted that there were twenty-eight children under a year old who received two or more injections. Of these eight were injected three times, four were injected four times, and one was injected five times. In none of these cases was any reaction obtained.

We have as yet obtained no material for histological examination from these human cases. A histological study of Arthus' phenomenon in rabbits has been made by Arthus and Breton[9] and by Thompson and Marchildon.[10]

CONCLUSIONS.

An immediate localized reaction of anaphylaxis in the nature of edema and infiltration with tenderness, followed in untreated cases by necrosis, occurs in human beings on reinjection with antitoxin. The percentage of cases in which this reaction occurs increases directly with the number of injections at short intervals given subsequent to a primary injection. This local reaction corresponds in all particulars to the Arthus' phenomenon in rabbits. The local reaction may or may not be associated with the immediate systemic symptoms described by v. Pirquet and Schick; as the number of repeated injections at short intervals (twenty-one days) is increased, the v. Pirquet and Schick reaction decreases in proportion to an increase in the local reaction. The discovery of this lesion completes the analogy between the serum sickness in human beings and the phenomena of serum anaphylactic intoxication in animals.

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MALIGNANT TUMORS IN MICE. WITH A REPORT OF A SPONTANEOUS ADENO-CARCINOMA IN A HOUSE MOUSE (MUS MUSCULUS).*

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Historical review. — The occurrence of malignant tumors among animals has been known for many years. It was not until Hanau[29] in 1889 attempted with some success to transplant a carcinoma in a rat to a healthy animal of the same species that the possibility of the artificial transplantation of malignant tumors attracted the attention of pathologists. Since that time, and especially during the last decade, a large amount of work has been done on the subject of the inoculable and spontaneous tumors in animals.

The first report of a tumor in a mouse was that made by Morau[41] in 1894. He succeeded in transplanting an adeno-carcinoma in a white mouse into other white mice of the same species. This work was followed by that of Loeb[38] in 1901 on transplantable sarcoma in rats. It is not within the province of this paper to consider tumors other than those occurring in mice.

The first important piece of research on tumors in mice was that done by Prof. C. O. Jensen,[36] of Copenhagen, and reported in 1903. His researches were entirely on white mice. He demonstrated that it was the implanted tumor tissue which underwent proliferation and not the tissues of the animal inoculated. His results were confirmed by Bashford[11] in 1895. However, there is another school which holds the view that the tissues of the animal inoculated also proliferate. The Jensen tumor (adeno-carcinoma in white mice) has been successfully transplanted through many generations of mice and is one which at the present time is still receiving careful study in this country and abroad.

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* Read by Dr. H. Gideon Wells before the Chicago Pathological Society, Jan. 11, 1909. Received for publication March 4, 1909.

The transplantation of the Jensen tumor is successful in forty to fifty per cent of the inoculations in white mice. Gray mice are much less susceptible and other animals are immune. Jensen[37] found that if the tissue was kept at 1° to 3° C. its ability to produce a tumor in an inoculated animal was not destroyed; if kept at 22° C. for eighteen days it still remained active and at 37° C. it was destroyed in twenty-four hours. Heating to 47° C. for five minutes also destroyed the ability to set up a tumor in an inoculated animal. Moore, Walker and Barrett, working in England, have found that continued freezing of the tissues does not inhibit the development of tumors in inoculated mice. Jensen also investigated the effects of intense sunlight and various disinfectants such as .25 per cent phenol, and found that five minutes exposure killed the tumor tissue. Apolant[2] has investigated the effect of radium rays on the tumor tissue and finds that they cause a retrogressive process in mouse carcinoma with a pronounced desquamation of cells. He has also, independently and with Ehrlich, studied the histology of the various mouse tumors very carefully.[3]

The adeno-carcinoma has been the most common tumor described in the mouse. This tumor has been found and transplanted only in hybrids and "sports" of the species *Mus musculus*. These tumors have been described and in some instances successfully propagated by Haaland,[30, 31, 32, 33] Borrel,[5, 6] Ehrlich,[20, 21, 22, 24, 25, 26] Tyzzer,[49, 50, 51] Michaelis,[43, 44] Bashford,[9, 10, 11] Loeb,[39, 40] Pick, Henke,[34, 35] Erdheim,[19] and some others.

Adeno-carcinomata have been most frequently observed in old female mice and it has been noted that they vary greatly in their ability to be transplanted. These tumors most frequently occur subcutaneously. The experiments of Ehrlich and Apolant[20] show that only about fourteen per cent of their primary tumors were transplantable, and that about forty to fifty per cent of the mice could be successfully inoculated. Ehrlich states that the sex and the age of the animal have no effect on the transplantability, and that passage increases the virulence of the tumors. Re-inoculation of mice is only successful in twenty per cent of the cases. Often in cases of inoculation with carcinoma the connective tissue elements also proliferate, giving rise to mixed tumors (sarco-carcinoma). On further transplantation of these mixed tumors Ehrlich found pure sarcomata developing. Loeb[39] has confirmed this observation in the tumors found in the Japanese waltzing mouse.

The question of the immunity of mice to malignant tumors has received some consideration by Ehrlich,[26] Clowes,[12] Michaelis,[44] *et al.* It has been noted that occasionally tumors after apparently being successfully transplanted, as is evidenced by increased growth, often entirely disappear and such mice are immune to further inoculation. Ehrlich inoculates mice with an avirulent hemorrhagic carcinoma and produces immunity in this way against virulent strains. Ehrlich has also shown that inoculation with attenuated carcinomatous material protects mice against sarcoma and *vice versa*. Various chemical agents, such as mercuric chloride, potassium cyanide (Clowes) and chloroform (Michaelis) have been used to attenuate virulent tumors and thus produce immunity in the inoculated mice.

The subject of the infectiousness and etiology of malignant tumors has been investigated to some extent in mice. It has been noted that carcinoma often develops spontaneously in healthy mice which are kept in cages with those inoculated or placed in cages after removal of such animals. Gaylord[27] in this country, Haaland[30] and Michaelis[48] have reported evidences of contagion of this kind. Gaylord[27] and Calkins[17] have reported the finding of spirocheta in mouse carcinoma. Blastomycetes and bacteria have been repeatedly found. Bashford[9, 10] states that he has found no evidences of infectiousness in the course of his experiments.

It should be noted in this connection that Clowes and Baeslack[14] have shown that the incubation (37° C.–41° C.) of tumors which normally produce very few transplants serves to increase this property, and obviously this suggests the similarity to the known infectious agents such as the bacteria and yeasts. They have, however, found in those tumors showing a large number of transplants that incubation serves to decrease this power.

Some phases of the chemistry of tumors have been worked out in mice. It has been demonstrated by Clowes and Frisbie[15] that rapidly-growing tumors in mice contain a high percentage of potassium and sodium and a small amount of calcium. It has also been shown by the same investigators that in old necrotic tumors the calcium content is high, with relatively little potassium. It is stated that the calcium, potassium and sodium content depend entirely on the rate of growth and the age of the tumor. Tumors showing necrosis and a high percentage of calcium are not inoculable. Often this necrosis is not due to bacteria, but to autolysis of the tumor cells. The exact nature of the action of these inorganic salts is not known, but it has been suggested that potassium and sodium when combined with the protoplasm of the tumor cells serve as stimulants in young active tumors, and that these substances are precipitated when the calcium of the old tumors combines with the complex cellular substance and produces an insoluble compound.

It may also be mentioned that Copeman and Hake[16] have investigated the amount of physiologically active hydrochloric acid in the stomachs of normal mice and those suffering with experimental cancer. They have found that it is decreased in the tumor mice.

Tyzzer[50] has made a partial study of heredity in relation to mouse tumors and finds that the offspring of inoculated mice develop tumors only in a small percentage of cases thus far noted. He thinks that it is possible that heredity may explain the occurrence of tumors among mice in certain cages, instead of cage infection. He states that there are not sufficient data to prove or disprove the hereditary transmission of malignant tumors.

Malignant tumors in mice are exceedingly important in that they furnish excellent material for the experimental study of the disease. While the majority of these malignant growths in mice possess some distinctive characteristics, yet there are some forms which show a striking resemblance to those occurring in the human body. Most of the spontaneous malignant growths in mice have occurred among " sports " and hybrids of the common mouse (*Mus musculus*) which have been kept in laboratories for experimental purposes. It is a well-known fact that hybrids, etc., are much more easily inoculated with tumors experimentally than the true species, which is only slightly susceptible.

Previous to 1904 no well authenticated cases of malignant tumors in wild animals were on record. We refer, of course, to wild animals in their natural state. A tumor was reported in this year (1904)[46] in a wild field mouse and is probably the first malignant growth to be described in a wild animal.

As has been stated previously, the majority of malignant tumors which occur in mice are among old females. The mammæ are probably the most frequent primary sites of infection and in addition the thyroid gland, lungs, and kidneys, with metastases often in the lymph glands, kidneys, and liver.

The mouse on which the subsequent report is based was first observed wild by Dr. Bayard Holmes, of Chicago, in his home and was later killed by him and presented to the Pathological Laboratory of the University of Chicago.

In reporting this case we believe that it is the first authenticated case in the literature of a spontaneous malignant tumor in a wild house mouse of true species (*Mus musculus*).

NECROPSY REPORT ON MOUSE (MUS MUSCULUS).

The mouse was killed by Dr. Holmes by chloroform and placed on ice. The necropsy was performed twelve hours after.

On examination the mouse proves to be the cómmon house species, *Mus musculus*. The animal, a female, is ninety millimeters in length to the base of the tail, twenty-five millimeters in width at the tenth rib (widest part) and weighs 23.2 grams.

The coat of hair is markedly roughened and is gray at many points, showing the animal to be quite aged. Kyphosis is present, the second to eighth thoracic vertebra being involved.

On external examination two tumorous masses are noted on the ventral surface. One in the left axillary region about the size of a small hazel nut and the other in the right and median mammary inguinal region (extra-abdominal) about the size of a hickory nut. The skin was carefully removed over the tumorous masses and the following measurements taken:

Axillary tumor	15 mm.	8 mm.
Mammary tumor . . .	27 mm.	17 mm.

The axillary tumor presents a very much lobulated appearance and is pinkish white in color, suggesting a carcinoma. The mammary tumor is dark and seems to contain blood, suggesting a hemorrhagic sarcoma. On section under strict asepsis the axillary tumor presents the same lobulated and opaque appearance. Cultures negative. On sectioning the mammary tumor there escapes about one-half dram of hemorrhagic pus. Cultures (twenty-four-hour) on mouse-blood agar show a pure culture of a micrococcus with a diplococcus grouping. After a somewhat longer period there also developed a species of saccharomycetes. The cultural and inoculation experiments with these organisms are discussed below.

One-half of each tumor was then excised and one-fourth placed in Zenker's fluid for microscopic examination and the remaining one-fourth placed on ice to be used for transplantation.

The further examination of the internal organs showed the following:

Lungs, normal; microscopic, normal. Liver, markedly enlarged and congested; microscopic, passive congestion; no metastases; Kupfer elements very prominent; cultures negative. Kidneys, dark in color, otherwise normal; microscopic, slight passive congestion; cultures negative. Intestines, normal. Thyroid, normal (?) (no microscopic examination). Uterus and ovaries, normal. Lymph glands, normal (probably not all found).

PATHOLOGICAL HISTOLOGY OF THE TUMORS.

Mammary. — The macroscopic appearance of this tumor has been described above.

Microscopic: This tumor presents a lobulated appearance and is essentially alveolar in structure. Rather heavy bands of fibrous connective tissue in all stages of development separate the lobules. Imbedded in this fibrous tissue in certain areas are small bunches of cells which resemble those of a young sarcoma. There is evidence of mixed infection and acute inflammation. Around the edges of the tumorous mass are found large numbers of polymorphonuclear leucocytes. In certain areas these leucocytes infiltrate the fibrous connective bands and in a few places the medullary portion of the tumor. In places large cavernous sinuses filled with blood are noted. These sinuses are present both in the fibrous connective tissue and in the medullary portion. The blood vessels of the tumor contain blood.

The central or medullary portion of the tumor is composed almost entirely of large cuboidal epithelial cells arranged in the form of alveoli. A single layer of cells lines each alveolus. The lumen of these alveoli is empty in some cases and in others filled with a substance similar in appearance to colloid. This substance, however, does not give the reactions of colloid with Van Gieson's stain. The stroma in the tumor proper is practically reduced to the smallest possible amount. Epithelial cells infiltrate nearly all the possible space between the intervening alveoli. A very small amount of fibrous connective reticulum carrying capillaries is noted. The nuclei of the cells lining the alveoli usually stain deeply, the chromatin being rather evenly distributed. Frequently the intervening epithelial cells do not stain as deeply as the foregoing. Some portions of the tumor show rather large cystic cavities lined in most cases with a single layer of epithelium similar to that which lines the smaller cavities of the alveoli.

The epithelium of some of the cysts is degenerating. Some of the cysts contain blood and others contain a homogeneous substance similar to colloid which has been referred to above. A very few karyokinetic figures are noted in the sections. Direct nuclear fission is the common method of division. In some ways the tumor resembles those primary in the thyroid, but the colloid material is absent. On careful examination the tumor is found to resemble one of mammary gland origin most closely, though it is not exactly typical. Certain tumors of the mammary glands of dogs and sheep resemble it very closely. We are inclined to believe that this is a primary tumor of the mammary gland of the mouse, although no normal gland could be found. The axillary tumor on the opposite side is small and probably metastatic. Such an occurrence has also been noted by Tyzzer.[52]

Diagnosis: Primary cyst-adeno-carcinoma of the mammæ.

Axillary.—The macroscopic appearance of the tumor has been described previously.

Microscopic: This presents the same type of epithelial cell noted in the mammary tumor. The heavy fibrous connective tissue bands which are prominent in the mammary tumor, encapsulating and dividing the tumor into lobules are not noted in this one. Small amounts of fibrous tissue are observed forming a delicate reticulum among the epithelial cells which compose the major portion of the tumor. This delicate network of fibrous connective tissue carries the blood capillaries. An alveolar arrangement with infiltrating cells is noted in all parts of the tumor. Acini are present in certain parts and contain a granular eosin-staining substance in some cases. The acinous-like structure is, however, often absent in the alveoli, the cells being closely packed together. Several large cysts are observed. The epithelial lining of the majority of these cysts is either absent or degenerating. In some cases the lining is composed of a single layer of epithelium, in others of several layers of closely packed cells.

With few exceptions these cysts are empty, these few containing a granular, pink-staining, albuminous substance. Karyolysis and pyknosis are noted. No karyokinetic figures are observed, direct nuclear division being the method of reproduction. No normal lymph gland was observed, but in all probability some remnant was present.

Diagnosis: Secondary cyst-adeno-carcinoma.

TRANSPLANTATION EXPERIMENTS.

Tissues from both tumors which had been kept on ice were sectioned as thin as possible, placed in sterile .85 per cent sodium chloride solution, and ground for a moment in a sterile mortar. The emulsion from each tumor was then carefully implanted subcutaneously into ten ordinary house mice of the same species and race as the original mouse. These mice were implanted on the thorax, in the inguinal region and sides of the abdomen. The results of these implantations were negative after three months. The mice are still under observation. One male mouse developed a small tumorous mass about the size of a pin head in the mid-abdominal line just below the ensiform cartilage and not in the exact region of the implantation. This mass has not increased in size since it was first observed and we attach no significance to it. No evidences of infection or inflammation were noted in any of the mice inoculated.

Implantations of the tumors into white mice and white rats gave negative results as was to be expected from previous work.

The fact that the mouse was killed with chloroform and also that no general karyokinesis was present possibly is the explanation for the failure to transplant this tumor.

CULTURAL CHARACTERISTICS OF MICROCOCCUS FROM MAMMARY TUMOR.

In the beginning the inoculations were made directly from the pus from the mammary tumor on agar smeared with sterile mouse blood. The bacterial growth which developed could not be transplanted to any other media without the addition of mouse blood. It has been possible of late to grow it on plain agar after carefully reducing the mouse blood added at each transfer of the culture.

The growth on the agar is grayish white in color and diffuses in a thin film over the surface of the media. The growth does not increase in amount after forty-eight hours. The colonies are round, grumose in the center, and under the microscope the edges appear slightly undulate.

Inoculations on plain bouillon, mouse blood bouillon, gelatin, potato, milk, blood serum (beef and horse) are negative. No acid is produced in litmus milk. The organism seems to be aërobic.

Microscopic examination shows a micrococcus with a tendency to diplococcus grouping. It stains well with the ordinary analin dyes and by Gram's method. The coccus measures about .3 micron in diameter in stained preparations.

Since the organism is entirely different from any before described and since it possesses the property of producing rapid septicemia in inoculated mice, we have named it *Micrococcus septicemiæ.*

Inoculations with micrococcus. — Five mice were each inoculated subcutaneously with .2 cubic centimeter of an .85 per cent NaCl emulsion of one-fourth of an agar slope culture, forty-eight hours old. All of the mice died of septicemia in twenty-four to thirty-six hours. The same species of micrococcus was isolated post-mortem. Another series gave the same results. Attenuated cultures (emulsion heated to 70° C. for one hour) gave no results when injected into a series of ten mice. No abscesses developed in any of the mice injected.

CULTURAL CHARACTERISTICS OF THE SACCHAROMYCES FROM MAMMARY TUMOR.

It will be recalled that this saccharomyces was in the beginning very slow in its development. A pure culture was obtained from the pus of the mammary tumor in conjunction with the micrococcus referred to above.

The organism was inoculated into solutions of dextrose, lactose, maltose, saccharose and mannite, in fermentation tubes. No fermentation or gas production was noted in any of the tubes, although the organism grew well. On potato and in milk no growth was observed. Gelatin is not liquefied and on stab culture the yeast shows a fine arborescent growth deep in the media along the line of inoculation and a small amount of clear white growth on the surface. The organism is a facultative anaërobe. Blood serum is not digested and only a slight growth occurs. The growth on agar is rather luxuriant, white in color, and capitate with a tendency to become umbonate. It grows exceptionally well on agar smeared with mouse blood. Colonies are reticulate with edges lobate-lobulate. Bouillon becomes cloudy and a heavy precipitate develops in twenty-four hours in the bottom of the tube.

Microscopic examination reveals a yeast of typical structure and average size. Some of the cells are budding. The saccharomyces stain with all the analin dyes and by Gram's method.

We have been unable to identify this yeast, but as yet it has not been named.

Inoculations with the saccharomyces. — The culture of the saccharomyces was grown for four days on an agar slope. Five cubic centimeters of a solution of .85 per cent NaCl was then added to the tube, the surface growth scraped off and an emulsion made.

Ten mice were injected subcutaneously each with .25 cubic centimeter of the above emulsion. Three died in twenty-four hours. The saccharomyces was not isolated from these animals, neither were there any other microörganisms found. Six other mice developed tumorous swellings in three days which gradually subsided without suppuration in five days more. Five of these mice are still alive and show no signs of any growth. Histological examination of one of these swellings shows an acute inflammation with leucocytic infiltration. The injection of .5 cubic centimeter of the above emulsion intraperitoneally produced toxic symptoms including dyspnea, nervous tremors, etc.

We are aware of the finding of the saccharomyces in malignant tumors by Sanfelice,[48] Roncali,[47] Aievoli,[1] Busse,[7, 8] Curtis,[18] and many others. For a period extending from 1895 to 1902 studies of yeasts isolated from malignant tumors were frequently reported. Brouha[4] has made some very interesting observations along the line of the immune bodies developed. Malvoz[42] had previously observed that agglutinins were developed in the serum of certain animals on inoculations with yeasts. Brouha in order to establish an etiological connection between carcinoma and yeasts applied a well known immunological principle and tested the agglutinin ability of the serum of individuals affected with carcinoma against two common and five pathogenic yeasts. No agglutination was observed. This is, of course, strong evidence against the saccharomyces as etiological factors in carcinoma. Nevertheless, on inoculation with yeasts mice frequently develop tumorous swellings. It has been determined after careful examination that in all probability these growths are distinctly inflammatory and not neoplastic. It must be borne in mind, however, that at certain times it is very difficult to distinguish between granulation tissue and some malignant tumors such as sarcoma.

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THE PATHOLOGICAL CYTOLOGY OF SURGICAL SHOCK.

I. PRELIMINARY COMMUNICATION. THE ALTERATIONS OCCURRING IN THE PURKINJE CELLS OF THE DOG'S CEREBELLUM.*

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WITH AN INTRODUCTORY NOTE ON THE PATHOLOGICAL PHYSIOLOGY.

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INTRODUCTORY NOTE.

The phenomena of surgical shock have been familiar to clinicians and laymen for many centuries, and have been clinically studied and classified by many of the ancient as well as modern surgeons. There is a general agreement as to its symptomatology and etiology. Much data pertaining to the pathological physiology have been accumulated. The symptoms of shock consist of a large variety of altered physiological functions; as an example, a railway train runs down a trespasser. He is mangled, frightened; he has lost no blood; he is in shock. The cause and the phenomena are familiar. The blood pressure is low, the respiration shallow, the muscles relaxed, the skin pale, cold, and moist, the intellect impaired. There has, in short, occurred a widespread change in the function of a large part of the cerebro-spinal axis. What, if any, physical change has occurred in the nerve tissue composing the part of the cerebro-spinal axis whose functions are thus altered? The cases that survive recover gradually, the blood pressure regaining the normal after many hours. The ultimate restoration of all the functions to the normal may require weeks or months, or even years. Are there no physical lesions associated with the

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* Received for publication March 8, 1909.

altered or destroyed functions? There has been heretofore no pathological histology of surgical shock. This pathological negation by methods hitherto employed is to be expected, since no one would suppose any disarrangement of the histological elements of the central axis. There could be no gross solution of continuity of the elements. The clue to a physical basis of the pathological phenomena must lie in a study of the cells by newer methods which will show physical alterations within the nerve cells corresponding to the physiological changes.

The pathological physiology that demands explanation by pathological cytology may be indicated by the following: surgical shock is the sum total of the intensity and number of stimuli, as well as of the number of nerve elements involved. A given number of stimuli of equal intensity applied to one neuron would not produce as much shock as would be produced if distributed over many neurones. The intensity of the shock, then, is the sum of the number and intensity of the stimuli and the number and type of the neurones involved. How much trauma is required to produce shock, and when may shock be said to exist? The central nervous system is in shock whenever it has been subjected to a sufficient afferent stimulation to produce pathological alteration of function. From the slightest, perhaps we may say infinitesimal, with evanescent effect, to the overwhelming and fatal shock the difference is in degree only. The number, intensity, and distribution of the stimuli plus the individual susceptibility make up each of the infinite number of the degrees of intensity of shock. It follows that if there is a demonstrable physical change in the elements of the central nervous system, such changes must correspond to the distribution and degree of intensity of shock. Furthermore, since the symptoms are the same in shock however produced, the microscopic changes should be found constant.

As previously stated, the entire cerebro-spinal axis is involved. Now this involvement includes the greater part of its functions. The function of paramount importance to the surgeon is that of the control of the circulation. Although other phenomena are important, they become insignificant in comparison with the loss of vasomotor power. Hence, the principal shock phenomena thus far studied by me is that relating to the circulation. Anemia is a powerful secondary factor in shock; but in an unpublished research we have shown that when the factor of anemia is totally eliminated by over-transfusion, shock, though much deferred, still may occur. All the experiments were planned so as to conform in type to the researches previously made by me. The pathological studies of Dr. Dolley form an addition to the work on pathological physiology heretofore done, and coördinates and supplements it.

The cytological study was begun in June, 1908. Since the attempt was inspired by Dr. Crile, and since, on account of our former close association, the natural hope is entertained that it may go far in substantiating his well-known views, the introductory note to show his connection with the work was requested. It is the intention to present in the course of time the results of the same methods of study applied to the remaining parts of the nervous system, peripheral and central.

SOURCE OF MATERIAL.—The dog was chosen for experimentation, since it is the most suitable animal for the study of shock and since the recorded data pertain largely to it. Few of the animals used had been confined more than ten days and all appeared healthy.

To produce the condition of shock, manipulation of the contents of the peritoneal cavity has been the only procedure so far employed. The intestines, conveniently drawn out through the abdominal incision, have naturally received the largest share of the manipulation. The treatment consisted in rubbing, sponging, and kneading the parts, with the intestines for the most part exposed to the air, and while the handling was naturally much severer than during a surgical operation, great care was taken to avoid laceration and the resulting hemorrhage. The operations were performed with due regard to asepsis. Ether was the anesthetic of choice, and the amount given was reduced to the minimum compatible with the complete physiological effect. Further, in regard to the amount of anesthetic required in the prolonged experiments, the experience has been that in animals shocked to death, the need for it progressively diminishes with the fall in blood pressure, and in the later stages, often quite one-third of the total time, none is required.

Such treatment, kept up fairly continuously, results in the death of the animal after an average time of slightly over three hours. This average was determined from eight cases, in which the minimum period of survival was two hours and the maximum four hours and twenty-five minutes. The maximum extreme, however, represents the attempt by means of more gentle handling and longer intermissions to delay the time of exitus for the possibly increased effect on the nervous system. The well-known appearances of shock which animals so treated exhibited need no comment. Manometer tracings of the blood pressure were taken in all cases to obtain an index of the condition of the animal.

In contrast with the immediately fatal experiments, milder variations of the condition were obtained by lessening the time of traumatic manipulation. These variations on the one hand consist of animals which after handling of the character indicated, lasting from thirty to fifty minutes, and after closure of wounds and recovery from the anesthetic, died within twenty-four hours and, on the other hand, of those in which after similar treatment there was a prognosis of presumptive recovery. These were killed for examination after times varying from six to twenty-four hours. Two dogs out of six treated for thirty minutes for this as well as other purposes died during the night, though three from thirty-three to forty minutes promised complete recovery. But the animals which survived all showed a marked susceptibility to ether, and three succumbed to a minimal amount at a secondary operation. Otherwise their condition was generally good. A few appeared somewhat weak and unsteady. Several blood-pressure tracings which were made showed a higher level of pressure than on the preceding day.

Neither in the fatal cases nor in those killed for examination was there the complication of definite infection. As would be expected after such treatment, the peritoneal surfaces were congested, though it was of low grade, and there was a slight serous exudation with perhaps a little fibrin. Not only was the time insufficient for the development of infection in most of the experiments, but several dogs similarly treated and in addition subjected to a secondary craniotomy or laminectomy failed to show any naked eye appearance of the development of bacterial inflammation within an ample period. It is therefore justifiable to assume that the aseptic precautions were sufficient and that death was due to shock alone. However, there seems no reasonable doubt that a non-infectious, traumatic peritonitis is caused, which continues to react abnormally on the nervous system for some time after closure of the abdomen.

Several other variations of the experimental procedure are in contemplation, which will be described at the appropriate time.

Whatever theory be held as to the nature of traumatic shock, there can be no doubt that the associated anemia resulting from the low blood pressure, especially in the severer forms, is an important factor in its manifestations. It is well established that acute partial anemia in hemorrhage is accompanied by chromolytic alterations in the Nissl substance, with its actual disappearance in severe cases,[1, 2, 3] while a total anemia such as is obtained in resuscitation after relative death, which has been studied by the writer,[4] results not only in chromolytic but also in structural changes indicative of cell death, particularly in the animals which subsequently died. Elimination of the anemia factor is therefore essential in any attempt to study shock as a separate entity. In the milder variations of experimentation just cited, no injurious fall of blood pressure occurs. With a longer manipulation, this difficulty may be obviated to a certain degree by taking the tissue before the blood pressure has fallen markedly, but it is open to the objection that it does not permit sufficient time for the accomplishment of a complete effect. Recent development of the technic of the transfusion of blood places at our disposal a means whereby blood pressure cannot only be maintained but controlled at will within its natural limits. As the nervous control declines, the pressure may be held up by the mechanical means of surcharging the vessels with blood. It is of course necessary to grade the amount to the ability of the heart and the weakened condition of the vessel walls. Dilatation of the heart, diapedesis of blood, rupture of splanchnic vessels, even actual rupture of the liver occurred in experiments in previous work. But when done slowly and carefully no untoward effects are apparent. The status of transfusion in relation to shock may be adequately expressed by saying that it is undoubtedly the most reliable therapeutic agency at our disposal. The technic consisted in making a carotid-jugular connection between a donor and the shock dog prior to any manipulation. Whenever blood pressure showed a tendency to fall, transfusion was made, and thereby the pressure was held very closely to the original level.

MICROSCOPIC TECHNIC. — Some of the tissue for examination has been taken *intra vitam*, while the greater portion was removed immediately after death. Material from animals dying during the night was not used unless the body was still warm.

Three routine fixing fluids are used, *i.e.*, ninety-six per cent alcohol, originally recommended by Nissl, a saturated solution of corrosive sublimate in ten per cent formalin, and in the later experiments, picrosulphuric acid, recommended by Held for his modification of the Nissl stain. The corrosive-formalin solution possesses all the value of a mercury fixative, with the quick penetration of the formalin. For small slices of brain tissue two to three millimeters thick, two to three hours are sufficient. The tissue is run through the graded alcohols without preliminary washing in water. The alcohols are iodized from the fifty per cent to the eighty per cent. The picrosulphuric material after twenty-four hours is also run through the graded alcohols. Imbedding is done in paraffin. The present article is based on the study of 5 μ sections.

The modification of the Nissl stain devised by Hans Held[5] has afforded the best results. Under "light" warming, the sections are stained for one to two minutes in a solution containing erythrosin, one gram, acetic acid, two drops, and water, one hundred and fifty cubic centimeters. After washing, they are stained under "strong" warming in a solution consisting of equal parts of Nissl's methylene blue soap solution and a five per cent aqueous solution of acetone "until the odor of acetone ceases to be given off." After differentiation in a one-tenth per cent solution of alum, they are transferred to absolute alcohol and cleared in xylol. It not only brings out the Nissl substance sharply and distinctly, but counterstains with exactness of detail the entire cell structure. Instead of heating the sections, the technic has been modified by staining in the second solution at a temperature of 37° C., usually for six hours, though a longer time makes no appreciable difference. Damar seems much superior to colophonium as a mounting medium. Staining at incubator temperature is also used for the Nissl stain in preference to strong heating.

The sublimate material is stained in bulk in Grenacher's borax-carmine, and differentiated in acid alcohol. Not only has it proved a reliable stain for the chromatic material, but it possesses the advantage that after study in a temporary clove oil mount, the same slide may be restained by Heidenhain's or by diluted Delafield's hematoxylin, to which the carmine becomes a counterstain. For this suggestion I am indebted to Dr. Howard.

THE CYTOLOGICAL ALTERATIONS IN THE PURKINJE CELLS OF THE CEREBELLUM. — Fourteen experiments form the basis of the present article, whose character is brought out in the course of presentation. So far only material taken from the worm of the organ has been studied. Tissue for blocks was cut sagitally.

Outside of the ordinary processes, any description of pathological alterations in nerve cells must concern itself chiefly with a discussion of the stainable substance of Nissl. That is true in the present case, but in addition to the finding of definite alterations, the interpretation of these changes appears to throw new light on the nature and origin of the Nissl substance. So strong does the evidence at hand appear that the description will be made in terms of the conception to which the findings have led. However, it is not the intention in the present series to deal other than objectively and incidentally with this aspect of the Nissl substance. It will be presented in separate form subsequently if more extensive data from this as well as other lines of experimental study which fully concord, so far as progress has been made, bear out the present hopeful indication.

Alterations in the amount and distribution of the Nissl substance are to be presented which have been interpreted as indicating that it is derived from the chromatic material of the nucleus. Not only does the intra-nuclear chromatin first increase, then decrease, and finally disappear, depending on the duration and severity of the experimental procedure, but the extra-nuclear chromatic material, the so-called Nissl substance, accompanies it, precedes it, or follows in its wake through the different stages in such a way that a definite relationship appears to be established between the intra-nuclear and the extra-nuclear content of the cell. The term "chromatic material" will be used as a general designation of the basic staining element whether inside or outside the nucleus.

This theory has a basis of support in preceding work. This is found in Scott's[6] investigations on the chromatic substance which forms the Nissl granules in the embryos of birds and mammals. His conclusion from this form of attack is that the granules are undoubtedly derived from the nuclear chromatin. The nucleo-proteid nature of the Nissl substance, suggested first by Held, supported by further work of MacKenzie and others, and confirmed by Scott, led to the inference that the nucleus is a possible source of the substance. Scott describes the process of origin in its essentials as follows, dividing it arbitrarily into stages:

First, in the germinating cells, the chromatin is confined to the nucleus and is in mitosis. Second, in the neuroblast stage, the chromatin is confined to the nucleus, but is broken into masses. A nuclear membrane has been formed and a greater part of the chromatin is distributed around the membrane. Third, some of the kinetic chromatin is transformed into two other kinds, an oxyphile and a basophile. As the chromatin alters, the basophile part diffuses into the cytoplasm, but the oxyphile substance remains in the nucleus. Fourth, the transformation of the kinetic chromatin into the oxyphile and basophile kinds is now completed, and the diffused basophile part fills the whole cell body. Fifth, adult cell, differing only from stage four in the distribution of the chromatin. One part of the chromatin does not alter and constitutes ultimately the peripheral portion of the karyosome.

The question as to whether the nucleus keeps on sending new material from the karyosome to the cytoplasm during the life of the cell, he leaves open. If it does, he is inclined to believe it is in solution and not in formed masses.

Richard Goldschmidt, in his lectures (Oral Communication of Dr. Howard), speaks of the analogy between the Nissl substance and chromidial apparatus.

CHANGES IN THE AMOUNT AND DISTRIBUTION OF CHROMATIC MATERIAL. — Alterations in the relation of the intranuclear and extra-nuclear chromatic material can be followed in a definite sequence. In these alterations are involved both deviations from an amount normal to the whole cell or to parts of it, and variations in the usual distribution. There may be an increase of the normal amount or there may be a decrease. Further, both phases may occur in the same animal. It is therefore essential to establish logically the priority of appearance between the increase and the diminution of chromatic material. To summarize the comparative study at the present point, an increase of chromatic material occurs in a large number of the cells of the animals subjected to a manipulation relatively moderate both as to time and to severity, while cells with a decrease are relatively scanty. On the other hand, all degrees of the diminution predominate in those animals which received a fatal handling. Since the procedure was identical in all cases, a short handling must result in stages earlier than does a prolonged one. Furthermore, a progressive increase of degenerative appearances is associated with the diminution of chromatic material, while such appearances are lacking in cells with a normal or increased amount. The priority appears, then, to belong to the increase of chromatic material, which is followed by a decrease.

Nissl early pointed out that there are not only variations which occur in the same type of cells under different conditions, but also that there are variations in the individual cells of a group under the same conditions. The limits of normal variation are therefore rather wide, and the point of absolute departure from the normal is usually difficult to determine. Originally with the idea of controlling this by comparison, material was obtained from eight normal dogs, some of which were killed quickly, while others were subjected to an ether anesthesia for fully as long as in the most protracted experiments. In the first place, the difficulty anticipated was not encountered for the reason that the alterations are very pronounced, all grades of diminution up to final and complete disappearance of both intra-nuclear and extra-nuclear chromatic material being present.

More important, however, the alterations found are interpreted as a forced perversion of the normal functional activities of the cell, which continues to respond until its capacities are exhausted; or, to express it more definitely, as the abnormal manifestation of the normal kern-plasma relationship. Regarded in the light of the relationship made apparent by the subjection to conditions potentially fatal, the normal variations above mentioned are made explainable as far as the working of the kern-plasma relationship is understood. The Nissl substance is put on a higher plane than the ordinary conception, which is even so low as to ascribe to it an adventitious character, and is made an integral structural element of the nerve cell. It is apparent not only from the definiteness but also from the interpretation of the alterations that the establishment of a normal mean was not necessary to determine any relative departure from it. The value of the control animals is only negative in failing to show more than a limited manifestation of the process described for the shock dogs, and no appearance at all of pathological alterations.

In taking up the subject of the distribution of chromatic material, only a brief reference is necessary to the distribution of the increased amount which appears to be the initial reaction. In cells showing an increase, the fact of an absolute increase is more important than the manner of its distribution, because the distribution corresponds in general to that described for cells under normal conditions. The chromatic material is in greater concentration. The relative number of Nissl granules may be increased, and usually there is fusion into larger masses. The intra-nuclear chromatic material is also increased, for not only does the karyosome stain intensely, but formed masses are found frequently, as well as the appearance of diffused chromatic substance, so that the oxychromatic nuclear material, which is usually so clearly defined by the Held stain, is obscured. When the nucleus lacks such an excess, the oxychromatic portion may be compact and solid, and stain a deeper red than is usual. Nissl's observations[7] concerning the relatively smaller size of such cells and the frequently irregular shape of both cell body and nucleus are fully apparent. While cells of such staining reactions occur likewise in animals under normal conditions, as has been pointed out by observers from Nissl down, the comparison of differential counts of the cells of normal dogs with those of the shocked dogs, in which the number of cells with increase of the chromatic material predominated over those with its diminution, shows that a considerably greater number of hyperchromatic cells occurs in such shocked dogs than in the normal controls. The diffuse staining reaction, which is more or less a feature of the majority of cells with increase of chromatic material, suggests the possibility of its presence in diffused, soluble form, both inside and outside the nucleus. This leads to the mention of cells so intensely hyperchromatic that the cellular structure is almost or entirely obscured. These "chromophile" cells of Nissl will form the object of special study.

There are three main stages in such cells as show a diminution of chromatic material:

First, the disappearance of the extra-nuclear chromatic material. Second, the using up of the diffused intra-nuclear chromatic material after it passes in turn into the cytoplasm, leaving the karyosome intact. Third, the giving up of the chromatic material by the karyosome, which after diffusion into the cytoplasm also disappears completely.

A description of the process with its intermediate stages may be given best by reference to the series of pictures presented. The disappearance of the chromatic material from the cytoplasm appears to begin at the periphery of the cell, which agrees with the usual description for the Nissl substance under pathological conditions. In Figure 2 a beginning of the disappearance from the dendrite is shown. However, as a fresh supply is continually coming to take its place, there is always more or less present in that situation in the stages exhibiting any extra-nuclear chromatic substance. Representing the first stage, all grades from a perceptible clearing up to complete disappearance of formed masses occur (Figure 3). With the completion of this stage, a change in the amount of intra-nuclear chromatic material is manifest in the way of an increase (Figure 4). It is massed up against the nuclear membrane, forming frequently a complete though irregular ring, so that the nuclear membrane may be entirely obscured. In more favorable cases, it is seen that sometimes the chromatic material is entirely inside; at others, both inside and outside this membrane. Irregular masses jut both ways from the membrane as a base. While such an arrangement so far as the nucleus is concerned may be seen in cells with general increase of chromatic material, its association in the present stage with entire absence of extra-nuclear chromatic material places it in a different category. Further, in the stages intermediate between this and a generally normal chromatic content, in which the disappearance of cytoplasmic chromatic material is becoming well-marked, there is generally no massing against the nuclear membrane of an excess of chromatic substance, and the distribution is well balanced, except where beginning to disappear. It gives one the impression of a final spurt on the part of the nucleus to make up for a totally inadequate distribution. From this point, the chromatic material massed about the nuclear membrane may be traced passing out into the cytoplasm (Figure 5). It is true that this results in a picture which at its inception is probably indistinguishable from the stage preceding the last, for the cytoplasmic content is again to a certain degree restored. But other cells are found which exhibit distinct differences. The most important is that the nucleus frequently suffers structurally. Actual breaks in the continuity of the nuclear membrane occur, and in several instances the curved-out ends at the point of rupture have been seen, as though it resulted from a force from within. Whether such appearances are the result of the process being described or are due to other conditions, which latter appears more probable, the point is that thereby the stage is differentiated. Without visible rupture, there may be also no visible demarcation between the nuclear and the cytoplasmic reticulum. However, other cells retain the nuclear membrane intact to the end. In addition, from the point of the preceding stage on, vacuolation and disintegration of the cytoplasmic reticulum begin to be apparent, which is also helpful.

However, it is the finding of cells belonging to the next stage (Figure 6) which gives the clue to what happens. That is a cell with all its structural outlines intact, but outside of the karyosome there is complete absence of chromatic material. The karyosome varies in its staining reaction, but commonly stains well, even intensely occasionally. With this exception the cell has used up all the chromatic material it could elaborate. The process then continues with the giving up of its chromatic content by the karyosome. A few early intermediate stages of this process have been observed. In the one depicted (Figure 7), the karyosome is distinctly outlined, but stains red instead of blue. Attached to the edge like a cap is a small amount of chromatic material, which has a distinctly dotted appearance. Further away on one side is a small mass having a fainter diffused stain. The only other chromatic material present is at the periphery of the cell toward the axone pole, where there are about nine granules, only three of which stain distinctly. Succeeding stages (Figures 8 and 9) in which the only vestige of chromatic material in the nucleus is a trace about the nuclear membrane, or in which it is entirely lacking, but in which a varying small amount is distributed in the cytoplasm, are readily found. The final result is a cell entirely devoid of chromatic material (Figure 10). The structural markings may be faintly recognizable, or they may be lost, and only the peripheral outlines, inclosing fragmentary material, be left with its dendritic attachment to mark out the site of the cell.

All the stages of the process just described, which is based on preparations fixed in alcohol, can be followed throughout in sublimate preparations stained in borax-carmine and hematoxylin. The intra-nuclear and extra-nuclear chromatic material stain equally well.

In order to gain a more definite knowledge of the relative number of cells of each type to be found and with the hope of correlating the data with the experimental variations, differential counts were made. One hundred consecutive cells were counted in each of the thirteen experiments so studied. Only cells appearing in their entirety were considered, which necessitated the skipping of many, so that no claim for exactness is made. It is felt, however, that if the data give an erroneous idea, it lies rather in being too conservative, because the most difficult cells to place are those near the last stage. With a nucleus obscurely outlined and possessing no chromatic material, but yet with such material distributed in the cytoplasm, they are revealed only by favorable section, and many apparently belonging to this category were not counted on account of insufficient evidence. More in order to state the findings concisely, stage six is taken purely arbitrarily to be the ultimate one with the possibility of recovery. Certainly this is questionable. In the five experiments of shocking the dogs continuously to death, an average of thirty-three cells out of one hundred were beyond the arbitrary recoverable stage, with a minimum of twenty-eight and a maximum of forty-six. Further, the number of cells just inside the recoverable limit (Figure 5) was relatively much greater than in the other experiments. The average number of cells with excess of chromatic material was ten and of those with normal distribution eighteen. To give a definite idea how the counts run, one will be given. Stage one, eight; stage two, sixteen; stage three, twelve; stage four, six; stage five, eighteen; stage six, eleven; stage seven, none; stage eight, two; stage nine, nineteen; stage ten, eight.

A striking difference is found in the milder variations of the experimental procedure. Of three animals after one-half hour's manipulation, killed respectively in six, twenty-one, and twenty-four hours, the first showed three cells beyond the recoverable limit, the second two, and the third none. Ten to fifteen per cent of the cells belong between stages three and five, while the rest are about evenly distributed between a normal and excessive amount. On the other hand, the same procedure fatal in about twenty-four hours gave fifteen cells beyond the limit and a well distributed count after the hyperactive stages which numbered forty-eight cells. And in an animal with intestinal manipulation for one hour and thirty minutes, which was killed for examination before the blood pressure had fallen seriously, twenty cells were beyond the limit, while fifty-seven contained a normal or excessive amount of chromatic material. The comparison of the cases cited shows a definite correlation with the experimental variations.

The only apparent discrepancy is in one dog, which survived the manipulation till the following day, when it succumbed to ether on attempting a secondary operation. The number of cells beyond the recoverable limit equalled the minimum of the fatal cases. A peculiar feature was the fewness of cells with normal amount of chromatin as compared with those showing an increase, the ratio being eight to forty-six. No note was made of his general condition, but the susceptibility to ether was exceptional and, in striking contrast to the usual experience, immediate attempts at resuscitation were absolutely futile. This discrepancy is probably not real and will be considered in later discussion.

STRUCTURAL ALTERATIONS. — In addition to chromatic alterations, structural changes are to be observed. They may be perceptible with the first disappearance of the extranuclear chromatic material. From there on they progressively increase in severity, though, as already stated, in some cells they may be but slightly marked throughout. In the cytoplasm, vacuolation is the principal feature. In addition, the lightening up of the contrast erythrosin stain in different areas or throughout the cell, and a distinctly more open arrangement of the cytoplasmic reticulum point to a general edematous condition. This is further substantiated by the increase in size by actual measurement. The dimensions of the cell may be doubled or tripled. In the extreme stages there is a partial or even complete disintegration of the cytoplasmic reticulum. A point which is approached with some hesitation is the apparent rupture of occasional cells in the final stages. It is common enough for the demarcation between the cell border and the surrounding tissue to be partially lost. Certainly, points of actual rupture occur (Figure 10). Whether they happen *intra vitam* or are due to the technic of preparation is left an open question. Sublimate fixation gives them as distinctly as does alcohol. When the enormous increase in size is taken into consideration, there is a priori no strong objection to such an occurrence. In the advanced stages of degeneration, an appearance of frequent occurrence is the condensation of the cell contents at one side, usually toward one or the other pole, while the opposite portion is entirely vacuolated or contains but a few shreds of reticulum. It occurs only in alcohol-fixed material and is most frequently seen at the periphery of the section. Finally, the condensation invariably occurs on the side of the cell farthest away from that which a penetrating fixing fluid would first meet. But it is a valuable index of the edema.

Of changes belonging to the nucleus, an eccentric position is of great frequency. The character of the cytoplasmic alteration gives sufficient explanation. The nucleus also becomes vacuolated and edematous and increases greatly in size. As already stated, the rupture of the nuclear membrane appears definite. The final stage is its complete disappearance.

Since variations in the size of cells are obviously so great, measurements were made of camera lucida outlines of cells and nuclei. This was done in four experiments. In three of them, both alcohol and corrosive-formalin preparations were separately investigated. Five cells of each type and after each fixative seemed to give a fair average. Comparison of the two fixatives shows that they correspond in a satisfactory way. The average dimensions of the alcohol-fixed cells are a little larger more frequently than smaller as compared with the sublimate preparations, but it appears to have no significance, for the differences were in no case greater than between individuals of the same set. Analysis of the statistics shows that the cells increase in size steadily through the early phases up to the point where the available intra-nuclear material is almost, or, in one case, quite used up, leaving an intact karyosome. From this point a progressive diminution in size through the remaining stages occurs. The increase is uniformly graded, with the exception that there is a sudden jump between cells with normal content and distribution and those showing well-marked disappearance of extra-nuclear chromatic material. That is to say, definite indication of disturbed kern-plasma relation is associated particularly with an increase in size. And the comparative suddenness of its appearance together with the more normal condition of the cells at this stage would point to a functional hypertrophy rather than a result of hydropic degeneration alone.

The nuclei increase in size along with the cells, though as far as the statistics go it is more uniformly graded. But they attain their maximum of size sooner than do the cells, at the point represented by Figure 4, when the whole nucleus is most active. Then they remain fairly constant through the two succeeding stages, but with the using up of the karyosome they decline, again accompanying the course of the cell.

COMPARISON OF MATERIAL FROM TRANSFUSED DOGS. — Three experiments in which the factor of anemia has been satisfactorily eliminated have been studied so far. Naturally those were selected for preliminary examination in which the manipulation of the abdominal contents was continued long enough to kill the animals despite the fact that the initial level of blood pressure was closely maintained. One animal was killed after two and one-half hours, the others died in two hours and ten minutes and four hours and twenty minutes respectively. The two-hour fatal experiment was upon a poor subject selected from necessity. This explains the difference in time — and perhaps the difference in the counts. In both cases blood pressure was held up to within ten minutes of cardiac failure which occurred rapidly.

It is sufficient to say that in all three experiments all stages of the process of disappearance of chromatic material are found, and the differential counts differ but slightly from the fatal cases with profound anemia for at least one-third of their course. Differential counts were made in the two fatal experiments. In the two-hour dog, thirty-seven cells out of one hundred were beyond the arbitrary recoverable limit, and in the four-hour animal twenty-five. In the latter animal the distribution of the remaining cells was more even than in the specimen count given, but in the former, cells with normal or excessive amount of chromatic material were very scanty. The evidence from these experiments and the one cut short before anemia occurred seems sufficient to show that anemia is not the predominant factor in the causation of the changes. Further efforts will be directed toward a more exact differentiation.

SUMMARY.

In brief, the following alterations are found in the Purkinje cells of the cerebella of dogs in various degrees of surgical shock produced by manipulation of the abdominal contents. There are deviations from the normal amount of the intra-nuclear chromatic material and of the extra-nuclear, the so-called Nissl substance, and variations in the usual distribution of both. There may be an increase or a decrease in the amount, and both may occur in different cells of the same animal, depending on the duration and the severity of the shock produced. The increase of the chromatic material appears to be the initial reaction, and is followed by a progressive decrease. The distribution of the increase of chromatic material is that of an animal under normal conditions. The distribution of the decreased amount can be followed in a definite sequence down to its complete disappearance, so that a close relationship appears to be established between the intra-nuclear and the extra-nuclear chromatic material. It is interpreted as indicating that the Nissl substance is derived from the chromatic material of the nucleus. The Nissl substance is considered as an integral structural element of the nerve cell. There are three main stages in the progressive diminution of chromatic material which form the basis of the conception:

First. The disappearance of the extra-nuclear chromatic material.

Second. The using up of the diffused intra-nuclear chromatic material after it passes in turn into the cytoplasm, leaving the karyosome intact.

Third. The giving up of the chromatic material by the karyosome, which after diffusion into the cytoplasm also disappears completely.

Differential counts of the number of cells in each animal belonging to these main and the intermediate stages afford a satisfactory correlation with the experimental variations.

Structural changes are also to be observed. The cytoplasm becomes edematous and vacuolated, leading to general disintegration of the reticulum, in which degeneration the nucleus shares. The cells may be doubled or tripled in size. In the increase in size, a functional hypertrophy appears to be a factor as well as hydropic degeneration, for the increase begins with a comparatively sudden jump in association with definite indication of disturbed kern-plasma relationship before degeneration is very noticeable. Appearances indicating actual rupture of the nuclear membrane and of the cell are not infrequently found.

The comparative study of experiments in which the factor of anemia was satisfactorily eliminated by the transfusion of blood from normal dogs shows the same succession of changes.

[I wish to express, though inadequately, my debt of gratitude to Dr. William T. Howard and Dr. Oscar T. Schultz of the Western Reserve University: to Dr. Howard for the freedom of his laboratory during vacation periods; to Dr. Schultz for the supervision of the preliminary microscopic technical work; to them both for going over the preparations and verifying the findings, and much enlightenment and many suggestions from their fuller store of cytological knowledge.]

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DESCRIPTION OF PLATES VI. AND VII.

(Purkinje cells of the cerebella of shocked dogs, preparations fixed in ninety-six per cent alcohol, imbedded in paraffin, cut at 5 μ, and stained by Held's modification of the Nissl stain; Reichert oc. 4, obj. 1/12 oil immersion, x 1,260, Spencer camera lucida.)

FIG. 1. Experiment Shock 11. — Cell with general increase of chromatic material of moderate grade, chosen because the structure is not obscured. Chromatic material both diffused and in formed masses appears within the nucleus. Since such cells adjoin others with little or no chromatic material, the appearance is not due to insufficient differentiation.

FIG. 2. Experiment Shock 31. — Cell with average normal amount and distribution of chromatic material in the cell body, though it is beginning to disappear from the dendrite.

FIG. 3. Experiment Shock 13. — Well-marked disappearance of chromatic material from the cytoplasm.

FIG. 4. Experiment Shock 19. — Almost complete disappearance of chromatic material from the cytoplasm, except about the nuclear membrane, where it is massed not only outside but also inside, and in greatly increased amount as compared with the preceding stages.

FIG. 5. Experiment Shock 13. — Excepting the karyosome, there is no chromatic material within the nucleus and very little outside. Otherwise, the secondary supply shown in the preceding figure has diffused out and been used up. The vacuolation and disintegration of the cytoplasm are very apparent.

FIG. 6. Experiment Shock 13. — The karyosome is the only chromatic element left.

FIGS. 7, 8, and 9. Experiment Shock 13. — Stages of the giving up of its chromatic material by the karyosome, with its diffusion into the cytoplasm.

FIG. 10. Experiment Shock 13. — Complete disappearance of chromatic material and of nuclear outlines. The appearance is definitely that of rupture of the cell at one point.

RHABDOMYOSARCOMA OF THE UTERUS; WITH THE REPORT OF A CASE.*

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Sarcoma of the uterus, in which varying quantities of striated muscle are found, are of rare occurrence. Many careful histological studies have been made of sarcoma uteri, but the literature yields only thirteen cases in which striated muscle also played a part.

It is evident from a careful comparison of these cases that tumors of this nature present a very uniform macroscopic picture. The tumor arises either within the uterus or upon the cervix, and invariably projects from the free surface. Those of the corpus uteri fill the uterine cavity, dilate the cervix, and project into the vagina. Those arising from the cervix fill and distend the vagina, and often spread up into the uterus, eventually filling its cavity. There is much greater tendency to project into the uterine cavity or vagina than to invade the walls of these organs. The surface of the projecting mass is coarsely lobulated, or consists of clusters of short, pedunculated, oval, grape-like masses of pinkish-red color, and firm or soft elastic consistence. Ulceration is usually very superficial, and does not generally occur until the tumor has reached a great size. Sarcomata containing cartilage, or myxomatous tissue, or fat, with or without the presence of striated muscle, present a similar macroscopic picture, thus the diagnosis of a rhabdomyosarcoma can be made only microscopically.

In common with other malignant tumors, early and profuse bleeding, and later a foul sanguino-purulent discharge, with anemia, pain, and emaciation occur, and the individual usually dies in exhaustion, with or without pelvic or abdominal metastases.

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* Received for publication March 9, 1909.

The case herein reported is as follows:

E. M. W.— Age, sixty-nine. Admitted to the Long Island Hospital Nov. 12, 1907.

Family history: Husband died of pulmonary tuberculosis; one child living and well.

Personal history: Health has always in general been good. Has borne several children; menopause sixteen years ago.

Present illness: About six months before entering hospital a slight bloody discharge from the uterus was first noticed. This soon became more profuse, but was unassociated with pain. When the bloody discharge from uterus first was noticed, the skin began gradually to darken, and this condition has gradually increased.

Present condition: A white woman of good development, but poor nutrition. Appearance decidedly cachectic. There is marked pigmentation of the neck, anterior aspect of chest, over the front of arms, and abdomen. The pigment occurs in discrete spots and blotches of deep brownish color, and is most marked over the chest and lower half of abdomen. The genitals are not pigmented. No markings of scratching. The roof of the mouth shows a diffuse pale yellowish discoloration, but no typical pigmentation.

Examination of chest and abdomen negative.

Pelvic examination reveals a reddish, hard, immovable mass about seven centimeters in diameter, filling the upper portion of vagina and covered with grayish exudate. The fundus is not palpable.

Clinical course: The patient soon became bed-ridden. The discharge became profuse, sanguino-purulent and very offensive and, on account of her weakened condition, operation was inadvisable. A month after entry to the hospital small, pale yellowish-brown spots appeared on the mucous membrane of the cheeks and palate; one on the right cheek was somewhat larger than a pin-point. The vaginal mass appeared to spring from the cervix, and increased much in size. The pigmentation increased. White count shortly after admission sixteen thousand one hundred.

The urine showed on an average a very slight trace of albumen and considerable pus, but no casts.

Six weeks after admission the blood examination showed:

Hgbl	50%
Red count	4,000,000
White count	16,800

A stained specimen showed:

No marked variation in size or shape of the erythrocytes, but marked pallor.

No marked poikilocytosis.

No nucleated reds.

No mast cells.

Differential count:

Polymorphonuclears	84%
Small lymphocytes	12%
Large lymphocytes	4%
No eosinophiles.	

Two months after admission the patient became completely bed-ridden and suffered much. Catheterization of bladder was necessary. Pain and restlessness was relieved by morphine. The temperature throughout varied. For the most part it was normal, or slightly elevated, but at times would rise to 101° F., towards the end it being subnormal. Pulse throughout was much increased, averaging from 100 to 110, and near the end 125. Death occurred two months after admission.

Autopsy 18 hours post-mortem.

Body: That of a white female, one hundred and sixty-one centimeters long; of good physique, and very fair nutrition. Pupils equal, measuring six millimeters. No palpable glands. Decubitus over sacrum. The skin of chest (especially over breasts) and abdomen is considerably pigmented, of brownish-black color, and distributed symmetrically and diffusely over both sides. The skin over abdomen is markedly wrinkled. Subcutaneous fat measures 1.5 centimeters in thickness over abdomen, and is of deep golden yellow color, and tough dry consistence. No edema of extremities.

Peritoneal cavity: Contains no free fluid or adhesions, except for a few loose tags attaching the great omentum to the spleen. Appendix appears normal. The pelvis is filled with a large tumor mass bulging up between the rectum and bladder.

Pleural cavities: The right side alone contains adhesions, which are entirely obliterating, and very dense. No free liquid.

Pericardial cavity: Free of adhesions; contains about fifteen centimeters of clear fluid. Serosa smooth and glistening.

Heart: Weight, two hundred and eighty grams. Myocardium of dark reddish brown color, and of rather soft consistence, and friable Chambers not dilated. Endocardium smooth. T., 11.8 centimeters; P., 7 centimeters; M., 8.4 centimeters; A., 7.5 centimeters; R. V., .4; L. V., 1.3 centimeters. Coronaries show a few patches of slight intimal thickening, but are patent.

Lungs: Cut surface wet and posteriorly shows marked congestion, in which are numerous slightly elevated grayish areas of firmer consistence, from which a turbid fluid can be expressed.

Liver: Weight, one thousand four hundred and seventy grams. Organ flabby, and on section the lobules are distinct, their centers slightly injected. Consistence normal.

Spleen: Weight, two hundred grams. Surface studded with firm white placques. On section the Malpighian bodies and trabeculæ are visible, and the pulp is pale and scrapes slightly.

Pancreas: Of good size, pale, and of firm consistence.

Gastro-intestinal tract: Negative.

Adrenals: Negative.

Kidneys: Weight, five hundred grams. Organs large and surrounded with abundant perirenal fat.

Left kidney on section shows a large dark pigmented calculus bearing in places rough spicules and in shape roughly forming a cast of the pelvis and calyces. The pelvic mucous membrane is injected, dull, and bathed in turbid yellowish fluid. The cortex is broad, pale, and not sharply demarcated from the darker medulla. There are no macroscopic abscesses. The glomeruli are just visible. The capsule strips readily.

Right kidney contains no calculi, but its pelvis contains turbid yellowish fluid. Otherwise its appearance is the same as the left.

Bladder: Thick walled, and contains thick yellowish foul fluid. The mucous membrane is wrinkled and in places deeply injected.

Uterus: The fundus is enlarged, measuring six centimeters in width, and five centimeters antero-posteriorly. Several small nodules project slightly from the serous surface of the fundus. The left tube and ovary appear normal. The right tube is visible where it joins the fundus, but its extremity and the right ovary are imbedded in the tumor mass. The lower portion of the corpus uteri and the cervix are the seat of a large tumor which projects into the vagina. The entire vagina is enormously distended, and its walls are drawn tensely over the tumor, though no part of its mucous membrane is invaded. The surface of the projecting mass is smooth, but undulating and coarsely lobulated. Its consistence is fairly firm, elastic, and slightly nodular. Its color varies from pinkish red to a dusky blue red. The most dependent part shows a small amount of superficial ulceration, in the center of which there is a small depression suggesting an external os uteri, but careful examination shows no distinct opening. The entire mass halved longitudinally shows no trace whatever of a cervical canal, or uterine cavity. The entire central portion of the mass is necrotic, soft, and in places diffluent. The cut surface of the large lower portion of tumor mass is mostly of grayish, but in places pinkish-gray color. The latter especially is seen near the periphery where macroscopically many small vessels can be seen. In places the cut surface is watery, and partly translucent. In the fundus the tissue is of deep russet brown color, and dry. The nodules seen on its surface, grayish on section, are sharply differentiated in color from the underlying russet brown tissue. There is no invasion of the bladder, rectum, or vaginal wall. The sacral and retroperitoneal lymph glands are not enlarged.

MICROSCOPICAL EXAMINATION.

Heart: Myocardial cells are well preserved, and slightly hypertrophied. There is slight connective tissue increase most marked about the vessels. The coronaries show some intimal thickening with some narrowing of the lumena. In places numbers of muscle cells contain numerous fine fat droplets.

Lungs: Large groups of alveoli neighboring bronchi contain an abundant fibrino-purulent exudate. The vessels in the alveolar walls are engorged — the bronchi are often devoid of epithelium, and filled with pus. The larger bronchi are entirely devoid of epithelium, the submucosa shows some connective tissue increase, with lymphoid cell infiltration, and marked engorgement of its vessels.

Liver: The hepatic cells show fair preservation; the sinusoids are engorged with blood, especially towards the centers of lobules. There are a few foci of necrosis, consisting of necrotic hepatic cells, and numerous polynuclear leucocytes. Some portal spaces contain groups of lymphoid cells. No connective tissue increase. Fatty infiltration very moderate.

Spleen: The Malpighian bodies are numerous, large, and irregular, and their lymphoid cells are of normal appearance. Their vessels show marked thickening with hyaline change, and perivascular connective tissue increase. The pulp sinusoids show marked engorgement, with considerable pigment within them or the adjacent tissue. The pulp connective tissue is increased. There are also some extensive hemorrhages into the pulp. The trabeculæ are thickened, and the capsule shows marked connective tissue increase.

(Pancreas and kidneys were not preserved.)

Adrenals: These are in good preservation. There is marked lymphoid and plasma cell infiltration of the medulla. The vessels throughout are deeply injected, and the adrenal cells of the medulla contain an abundance of yellowish-brown pigment (iron-free) disposed as fine irregular granules within the cells. The cells in places are separated by edema.

Bladder: Walls thickened; muscle hypertrophied. Connective tissue increased throughout. The epithelium is absent, and the connective tissue beneath is thickened, contains numerous small engorged vessels, and numerous foci of lymphoid and plasma cells.

Skin: A section of skin from vault of mouth shows small areas in which the epithelium and subcutaneous tissue is infiltrated with lymphoid and polynuclear cells. There is no pigmentation of the epithelium. A section of skin clinically pigmented shows a well-marked pigmentation of the basal layer of cells.

TUMOR: Sections from different parts show wide variations in cell types, and also in the amount of and distribution of the stroma. In general the tumor is very cellular, the stroma relatively small in quantity, though in some places it predominates, and in parts is markedly edematous. Towards the periphery of the tumor the cells often are arranged in alveoli separated by coarse conective tissue trabeculæ. Vascularity is a prominent feature, and more especially is seen in that part which projects into the vagina.

The entire central portion, which in gross appears necrotic, consists of deep eosin-staining tissue, at the periphery of which the outlines of cells with their nuclei are still visible, the latter staining very faintly, and indefinitely blue. The greater portion of this necrotic tissue is uniformly eosin-staining and granular, showing slight but distinct evidence of the former tumor tissue in that faint outlines of cells, and sometimes the large nuclei identical with those of the living tumor cells, are seen. The demarcation between necrotic and living tissue is sharply defined by a more or less thick, dense layer of connective tissue, from which small strands and large trabeculæ of connective tissue spring, to penetrate the living tumor. Again, isolated groups of tumor cells are enmeshed in the dense limiting connective tissue layer. A section from one part of the periphery shows small cells infiltrating smooth muscle, though this cannot definitely be recognized as uterine tissue. The vaginal surface of the growth is coarsely undulating, and in places lobulated, shallow crypts separating the lobules. The crypts are about six to eight millimeters deep. The exposed vaginal surface of the tumor is entirely devoid of epithelium, shows superficial areas of necrosis, and is covered in places with a thin purulent exudate. Close to this surface there are foci and diffuse areas of infiltration with polymorphonuclear and eosinophilic leucocytes. The protected surface in the crypts is covered in places with epithelium, often disposed in a single layer, or in two or three layers resembling transitional epithelium. The tumor cells vary widely in size and morphology. Roughly, they can be divided into two groups as follows:

(1.) Large, oval, or round cells, about half of which contain two or more usually deeply-staining nuclei, the latter varying widely in depth of staining. The multinucleated cells contain on an average two to six nuclei, though there are occasional cells containing fifteen to twenty. The nuclei may be centrally, or excentrically situated, bear no uniform ratio in size to that of the cell body, and in the multinucleated cells they often differ somewhat in size. The average diameter of this large type of cell is thirty-six microns, and that of its nucleus twenty-five microns; the largest cell observed measures fifty microns, and its nucleus thirty-eight microns. The nuclei are usually spherical, with evenly rounded, or slightly crenated edges. They contain a single, sometimes two or three nucleoli about three microns in diameter, which stain characteristically purplish after careful differentiation with the eosin and alkaline methylene blue stain. The chromatin is abundant, is distributed diffusely, or in large granules, but is mostly seen near the periphery of the nucleus. The latter sometimes contains one or more vacuoles, and occasionally there is a narrow clear margin about the nucleoli. In a few nuclei small or large single eosin-staining hyaline bodies are seen, sometimes lying within a vacuole. Occasionally they are very large and occupy nearly the entire nucleus. The protoplasm appears either as a wide unbroken sheet, limited by a definite cell membrane, or it is more or less vacuolated, or entirely surrounded by a clear zone, without which lies a wrinkled cell membrane. Often, however, delicate strands of protoplasm extend from this central mass to the cell membrane, thus giving to the periphery of the former a serrated appearance. In many cells fine eosin-staining lines in the protoplasm encircle the nucleus in concentric rings of more or less regularity, thus giving somewhat the appearance of an onion in cross section. A few of these cells also show definite striations radiating from the nucleus, intersecting the encircling lines. Often the protoplasm contains numerous scattered dots, which in places are seen to arrange themselves in lines so as to form definite striations. Rarely the cell membrane also is striated, and sometimes the delicate protoplasmic processes uniting the central mass to the cell membrane are striated. Some parts of the tumor are composed almost completely of these large cells, many of which are multinucleated or vacuolated or both, but only a very few show definite cross-striations. Rarely a large cell with multiple mitotic figures therein is seen, but none of these show striations.

(2.) Large spindle or cylindrical cells fairly uniform in size and averaging eighteen microns in diameter. The nuclei are relatively large, measuring sixteen microns in diameter, and often are sufficiently large to produce a distinct bulging of the cell wall. They are usually single, and of regular oval shape, though often considerably distorted. When two or more nuclei are present mutual pressure obviously accounts for the distortion. The chromatin is rather diffusely distributed, giving to the nuclei a vesicular appearance. One or more characteristic nucleoli are seen usually. The protoplasm is non-vacuolated, stains very faintly, and often the cell boundary is very indefinite. In longitudinal section the cell on either side of the nucleus often appears to be hollow, the protoplasm lying as a thin rim, sometimes striated, against the cell membrane. Thus the nucleus often appears to lie within a hollow tube, usually causing a marked bulging of the latter. The cell membrane is often distorted, or markedly wrinkled, and usually tapers to eventually become a single coarse dark fiber at either extremity of the cell. Occasionally the membrane shows definite transverse striations. A cross section of one of these spindle cells, other than through the nucleus, usually shows more or less oval mass of protoplasm with a hollow center, but often the wrinkled cell membrane alone is seen. Sections through the tapering extremities show only darkly-stained irregular or oval dots. Numerous mitoses are seen, but in none of these can any striated protoplasm be seen. At the periphery of the tumor the spindle cells are usually smaller, more closely arranged, and the nuclei are more regularly oval; the chromatin takes a deeper stain, and is more evenly distributed. The protoplasm is non-vacuolated and the cell margins are difficult or impossible to define. Thus, protoplasm and intercellular substance are difficult to differentiate. Amongst the smaller spindle-cells the mitoses are numerous. Some parts of the tumor are composed almost entirely of the large vacuolated oval cells; others are composed alone of spindle cells, whilst the usual picture is a mixture of both in varying quantities. The intercellular substance varies in quantity; between the large cells it is abundant, homogeneous, and stains precisely like the embryonic myxomatous tissue of the umbilical cord. There are no fibroglia or myoglia fibrils.

Reported by	Age.	Pregnancies.	Site, Character, and Progress of Primary Tumor.	Metastases.	Termination.	Microscopical Findings.
1. Weber, 1867.	45	9	Small, soft, reddish polyp on anterior cervical lip. Recurred several times after operation. Finally invaded body of uterus.	None.	Perforation of uterus. General peritonitis. Death.	Long spindle cells; smooth muscle. Striated muscle cells resembling embryonic striated muscle.
2. Anderson-Edmansson, 1870.	50	0	Arose from corpus uteri. Tumor large, soft, and nodular. Hysterectomy.	?	Death from hemorrhage.	Small mononucleated, and large multinucleated cells. Cystic glands. Typical striated muscle.
3. Kunert, 1874.	35	0	Soft, red polypi on cervix. Amputation of cervix. Later, nodules appeared in pelvic connective tissue which were removed.	Vaginal wall, pelvis, and pleura.	Death from thrombosis of iliac vein.	Large round cells. Striated muscle.
4. Bystroumoff and Eckert, 1874.	?	?	Small pedunculated tumor projecting from uterine wall.	None.	?	Small spindle cells. Large multinucleated cells. Striated muscle.
5. Colomiatti, 1882.	50	?	Tumor probably arose from corpus uteri. Size of fetus' head.	?	?	Large round cells. Striated muscle.
6. Pernice, 1888.	17	0	Arose from cervix uteri; later it invaded the body of uterus.	Abdominal.	Death from pneumonia.	Soft edematous and myxomatous connective tissue. Young striated muscle. Hyaline cartilage. Glandular elements.
7. Richter, 1892.	2½	—	Arose from cervix uteri. Two operations for recurrences.	+	Death after two years.	Round cells. Myxomatous tissue. Striated spindle cells.

Reported by	Age.	Pregnancies.	Site, Character, and Progress of Primary Tumor.	Metastases.	Termination.	Microscopical Findings.
8. Pick, 1894.	2½	—	Soft polypoid growth from posterior wall of cervix. Growth rapid.	Extension into uterus.	Death in cachexia.	Large round cells. Striated muscle.
9. von Franqué, 1899.	40	11	Large, soft, size of man's head. Arose from posterior uterine wall. Operation.	None.	Death one week after operation. Cachexia.	Striated muscle. Cystic glands. Spindle cells (sarcoma).
10. Gebhard, 1903.	56	12	Fist-size. Arose from posterior uterine wall and fundus. Pedunculated. Hysterectomy.	?	?	Spindle cells (sarcoma). Hyaline cartilage. Fat. Smooth muscle. Striated muscle.
11. Penkert, 1905.	62	12	Arose from anterior uterine wall. Pedunculated.	?	?	Spindle cells (sarcoma). Myxomatous and fat tissue. Hyaline cartilage. Smooth and striated muscle.
12. Läwen, 1906.	60	—	Posterior uterine wall. Hysterectomy.	Abdominal.	Perforation of uterus. General peritonitis. Death.	Spindle cells (sarcoma). Smooth and striated muscle. Large multinucleated cells. Glandular elements.
13. Hunziker, 1907.	58	2	Arose from anterior uterine wall. Surface nodular; rapid cachexia.	Pelvic involving rectum and bladder.	Death from cachexia.	Myxomatous tissue. Cartilage. Smooth and striated muscle.
14. Robertson, 1908.	69	2 +	Probably primary in uterus. Pinkish, soft, lobulated, and vascular. No operation.	None.	Renal calculus. Pyelonephritis. Death.	Spindle cells. Large multinucleated cells. Striated muscle. Myxomatous tissue.

The fourteen reported cases of uterine tumors (see table) in which striated muscle elements have been found all showed the clinical picture of malignancy. Most of them recurred after operation and ended fatally within a few months. All the tumors contained more or less rapidly-growing round or spindle cells, some described as smooth muscle. Seven cases also showed other elements, such as cartilage or myxomatous tissue, and in seven there were large round, often multinucleated, non-striated cells. An examination of the numerous reported cases of sarcoma uteri show that some or all of these elements (except striated muscle) are very frequently found, and that the external appearance is usually identical with that of the rhabdomyosarcomata. In fact Pfannenstiel[1] under "Das Traubige Sarcom der Cervix Uteri" includes in his twelve collected cases three which contained striated muscle; regarding the muscle as of no particular significance. It is also probable that striated muscle has not infrequently been overlooked where these elements are few in numbers. Thus it seems that striated muscle is not an essential part of these malignant tumors, but that we are dealing with a type of mixed tumor composed of embryonic elements, some cells of which occasionally differentiate into striated muscle, showing all stages of development up to that of the adult striated cells. Nehrkorn[2] found the latter in a slight thickening in the lower part of the posterior wall of an otherwise normal organ, and Wilms[3] believes that rhabdomyomata are mixed tumors arising from the mesodermal tissue, displaced from the lumbar region by the müllerian duct in its descent early in embryonic life. Certainly this theory is very plausible, as rhabdomyomata of many parts of the lower projection of the genito-urinary tract have frequently been found, *i.e.*, in the corpus and cervix uteri, vagina, ovary and testicle (Benaniti[4]). A recent, very careful study of mixed tumors in this situation is given by Kehrer.[5] They all show striking similarity in gross appearance, development, and microscopy.

SUMMARY.

The case herein reported is identical in gross and microscopical appearance with other cases previously reported. As it was not found until it had reached a large size, the point of origin is uncertain, but it appears to have arisen from the lower portion of the corpus uteri. It is very vascular, contains much myxomatous tissue, spindle and large round, single or multinucleated elements, a very few of which present striations.

Though by means of Mallory's fibril stains[6] many of the spindle cells show fine or coarse peripheral longitudinal fibrils, which often taper and fuse at the cell extremities, one cannot positively identify these as smooth muscle cells, because very occasionally the coarse fibrils contain definite transverse striations. It is of interest to note also the close resemblance that the larger oval or round vacuolated striated cells bear to the characteristic "spiderlike cells" first described by Cesaris-Demel[7] and lately by Wolbach,[8] who shows conclusively that the vacuoles lie entirely within the cell.

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DESCRIPTION OF PLATE VIII.

FIG. 1. — Periphery of tumor showing spindle cells invading smooth muscle. x 450.

FIG. 2. — A section taken from near the periphery of the tumor showing both varieties of cells. x 450.

FIG. 3. — Here the large vacuolated or oval cells and the spindle cells are about evenly mingled, and a large spindle cell shows striation of its protoplasm. x 2,000.

FIG. 4. — A large vacuolated cell, the nucleus of which is surrounded by a striated mass of protoplasm. x 2,000.

FIG. 5. — Spindle cells cut in various planes, some of which are striated. x 1,500.

EFFECT OF DILUTION UPON THE FLOCCULATION OF COLLOIDS. II.*

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In our previous communication on this subject[1] we showed that in flocculation of a colloid by an electrolyte there is no relation between the concentration of the electrolyte and that of the colloid. The lower limits of flocculation are the same no matter what may be the concentration of the colloid, and the process may be regarded as a "salting out," taken in a sense wide enough to include the acids.

The colloids dealt with in Part I. were inorganic, such as colloidal platinum, arsenic sulphide, and ferric hydroxide; organic, such as mastic; organized, such as bacteria. None of these colloids are in the form of salts, and all show the electrolyte or salting out type of flocculation with acids, or salts of the mono and divalent bases.

On testing certain protein solutions, however, in which the proteins are presumably in the form of sodium salts, we find that treatment with acids results in irregular series. Our attention was first directed to this phenomenon on testing laked red cells with hydrochloric acid with results shown in Table I., A.

Table I.

Flocculation of laked red cells by acid.

HCl.	A. Laked Red Cells, Dilutions of "Orig."				HCl.	B. Digested Red Cells, Dilutions of "Orig."			
Final Dilutions.	1/4	1/12	1/25	1/50	Final Dilutions.	1/4	1/8	1/16	1/30
Normal	+++	+++	+++	+++	Normal	+++	+++	+++	+++
1/10	−	−	−	−	1/10 to 1/1000	−	−	−	−
1/200	+	−	−	−	1/1500	+	−	−	−
1/500	−	+	−	−	1/3000	+	+	−	−
1/1000	−	−	+	−	1/6000	−	+	+	−
1/2000	−	−	−	+	1/12,000	−	+	+	+
1/4000	−	−	−	−	1/24,000	−	−	+	+

"Orig." = Original solution.

* Received for publication March 9, 1909.

In Table I., A, we find that with HCl at normal there is complete flocculation throughout the series irrespective of the strength of the colloid. The precipitate is very heavy and the fluid above clear and colorless. Evidently the protein constituents of the laked cells have been completely "salted out." From N/10 to N/100 HCl there is no flocculation, but at N/200 there is flocculation at 1/4 dilution of the colloidal solution, and the flocculation zones descend in proportion as the colloid is diluted. Within this second flocculation zone, however, the precipitation is very incomplete, and the supernatant fluid is red, while the precipitate is colorless. It appeared to us probable that there had been a chemical reaction between the globulin salts of the laked cells and the hydrochloric acid, in accordance with the equation, $HCl + Glob.\ OONa = NaCl + Glob.\ OOH.$, leading to the precipitation of the insoluble globulin acid, the hemoglobin being left in solution. To test this point the globulins were extracted from a solution of laked red cells by half saturation with ammonium sulphate. The filtrate contained the hemoglobin which was then salted out by saturated ammonium sulphate, the precipitate dissolved in distilled water, and dialyzed for a week. Table II. shows that with such a solution there is no lower or chemical reaction zone of flocculation, but merely the electrolyte type of flocculation with HCl at normal.

TABLE II.

Flocculation by acid of laked red cells after removal of the globulins.

HCl.	Laked Red Cells, Dilution of " Orig."		
Final Dilutions.	1/2	1/10	1/24
Normal	+++	+++	+++
1/10	—	—	—
to			
1/10,000	—	—	—

Since it was possible that the lower zone of flocculation shown in Table I., A, had been due to lecithids, or other ether soluble contents of the laked cells, a portion of dried cells was thoroughly digested with three changes of ether at 40° C., dissolved, and tested with HCl. The lower zone of flocculation appeared as shown in Table I., B, so that the possibility of the lower zone being due to lecithids was excluded. The original dilution of the digested red cells was much weaker than that of the undigested cells, so that less HCl is needed in B than in A to cause flocculation, but the type of the second flocculation zone is similar in character in both instances and is undoubtedly due to the same constituent. The ether extract of the laked cells suspended in water was not flocculated at all by hydrochloric acid.

Flocculation of serum. — Ox serum dialyzed one week. There is a considerable precipitate of globulins from which the clear fluid above is decanted off. On testing the fluid with half saturated ammonium sulphate there is a heavy precipitate, showing that the globulins, as measured by ammonium sulphate, had only partially separated out in the dialysis. Table III. gives the results of tests made with hydrochloric and sulphuric acids. The upper or salting out zone is not well marked but the lower or chemical reaction zone shows very clearly, the heavy type + + + indicating the points of optimum flocculation. It may be observed that at each dilution of the colloid, precisely the same strength of each acid is necessary for optimum flocculation, and on halving the concentration of the colloid the concentration of each acid at the optimum point is also exactly halved. These facts seem to indicate that the reaction is a chemical one. The flocculation zones in each case are rather wide, but there was no difficulty in determining the optimum points, *i.e.*, the points at which flocculation first began, and was most marked in the earlier stages of the experiment.

TABLE III.

Flocculation of serum by acids.

	A. Hydrochloric Acid.					B. Sulphuric Acid.				
Acids.	Serum, Dilutions of "Orig."					Serum, Dilutions of "Orig."				
Final Dilutions.	1/2	1/4	1/8	1/16	1/32	1/2	1/4	1/8	1/16	1/32
Normal.....	+	+	+	+	+	+	+	+	−	−
1/10.......	−	−	−	−	−	−	−	−	−	−
1/100......	++	−	−	−	−	+++	−	−	−	−
1/200......	+++	++	−	−	−	+++	+++	−	−	−
1/400......	+++	+++	+	−	−	+++	+++	+++	−	−
1/800......	++	+++	+++	+	−	++	+++	+++	+++	−
1/1500.....	+	+	+++	+++	+	−	+	+++	+++	+++
1/3000.....	−	−	+	+++	+++	−	−	−	+++	+++
1/6000.....	−	−	−	−	+	−	−	−	−	+

We have also tested another sample of ox serum prepared as follows: After dialysis in the presence of chloroform there is a very heavy deposit of globulin. The serum with its insoluble deposit is thoroughly shaken, one hundred cubic centimeters measured out, and treated with one hundred cubic centimeters of sodium hydroxide at N/150, which preliminary tests had shown to be the necessary strength for complete solution of the globulins without the fluid showing alkalinity to litmus. We have, then, the entire serum in neutral solution with an inappreciable amount of free electrolytes. Cultures showed the serum to be sterile, so bacterial influence can be excluded.

TABLE IV.

Flocculation of entire serum by acids.

	A. Hydrochloric Acid.			B. Acetic Acid.			C. Oxalic Acid.		
Acids.	Serum Dilutions.			Serum Dilutions.			Serum Dilutions.		
Final Dilutions.	1/2	1/4	1/8	1/2	1/4	1/8	1/2	1/4	1/8
Normal......	+	+	+	–	–	–	–	–	–
1/10........	–	–	–	–	–	–	–	–	–
1/100.......	–	–	–	+	–	–	–	–	–
1/200.......	+++	–	–	+++	–	–	+++	–	–
1/400......	+++	+++	–	+++	+++	–	++	+++	–
1/800.......	–	+++	+++	–	+++	+++	–	++	+++
1/1500......	–	–	+++	–	–	+++	–	–	+
1/3000......	–	–	–	–	–	–	–	–	–

Table IV. shows that in the lower zones the optimum points for the precipitation of the serum by acetic and oxalic acids are precisely the same as with hydrochloric acid. Unless the reaction is of a chemical nature, one would hardly expect the weak acetic acid to have the same effect as the strong mineral acids. In salting out, as has been maintained by Spiro[2] and Hardy,[3] the reaction is probably a physical separation into two phases, a solid phase containing much protein, little salt and very little water, and a fluid phase containing much water, most of the salt, and very little protein. Extending this hypothesis to cover the acids, we find, indeed, from Table IV., that the strong hydrochloric acid at normal salts out the serum proteins, but the weaker acetic acid does not. Among the mineral acids themselves, according to Walker,[4] nitric and hydrochloric are about equally strong acids, while sulphuric is only one-half the strength of either of these two. We have always found sulphuric to be much less efficient in salting out than either nitric or hydrochloric acid. When, however, it becomes a question of the chemical activity of the $H^{\bullet}$ ion, it is equally effective whether it is derived from a weak or strong acid.

It appeared from the completeness of the flocculation of the serum at the optimum points that both globulins and albumins had been carried down and treatment of the supernatant fluid with ammonium sulphate gave rise to a very slight precipitate. It remained, however, to determine if each component would be precipitated out of itself, or if the globulin on precipitation simply carried down the albumin with it. Dr. Banzhaf, of the New York Health Board, kindly provided us with globulin and albumin of horse serum to test this point.

The globulins were received as a precipitate containing much ammonium sulphate and were therefore dialyzed four days against tap water, and four days against frequent changes of distilled water. Suspensions were made in distilled water and tests made with acids. The suspensions, however, were not sufficiently stable and precipitated out spontaneously, so a very thick suspension was treated with an equal amount of sodium hydroxide at N/100. The globulins with this strength of alkali almost all dissolved, and were filtered; the filtrate, called original ("orig."), being neutral to litmus and somewhat opalescent.

TABLE V.

Globulins treated with acids.

	Hydrochloric Acid.				Nitric Acid.			
	Globulins, Dilutions of "Orig."				Globulins, Dilutions of "Orig."			
Final Dilutions, Acids.	1/4	1/8	1/16	1/32	1/4	1/8	1/16	1/32
Normal	+	+	—	—	+++	+++	+++	+++
1/10 to 1/400	—	—	—	—	—	—	—	—
1/800	+++	—	—	—	+++	—	—	—
1/1500	+++	+++	—	—	++	+++	—	—
1/3000	++	+++	+++	—	—	+	+++	—
1/6000	—	—	—	+++	—	—	+	+++

Albumins. — The albumins were received as the filtrate from horse serum after precipitation by half saturated ammonium sulphate. Crystals of ammonium sulphate were added to saturation and the resulting precipitate of albumins collected on a filter, dissolved in distilled water and dialyzed.

Table VI. gives the results with three acids to show the exact correspondence in strength of each acid required for the flocculation of the albumin, indicating a chemical reaction.

TABLE VI.

Flocculation of albumins by acids.

	Albumin, Dilutions of "Orig."								
	1/4			1/8			1/16		
Acids, Final Dilutions.	HNO_3.	HCl.	H_2SO_4.	HNO_3.	HCl.	H_2SO_4.	HNO_3.	HCl.	H_2SO_4.
Normal........	+++	+	–	+++	+	–	+++	+	–
1/10 to 1/200..	–	–	–	–	–	–	–	–	–
1/400.........	–	–	–	–	–	–	–	–	–
1/600.........	+	+	+++	–	–	–	–	–	–
1/800.........	+++	+++	+++	–	–	–	–	–	–
1/1000........	+++	+++	+++	–	–	–	–	–	–
1/1500........	–	–	–	+++	+++	+++	–	–	–
1/2000........	–	–	–	+++	+++	+++	–	–	–
1/3000........	–	–	–	–	–	–	++	+	+
1/4000........	–	–	–	–	–	–	+	+++	+
1/5000........	–	–	–	–	–	–	+	+++	+

Nucleo proteids. — Sheep kidney nucleo proteids in alkaline solution provided by Dr. Shaffer were reprecipitated with acetic acid, the precipitate washed, dialyzed, and finally redissolved in sodium hydroxide to neutral solution.

The table (VII.) shows the usual salting out zone at normal and the chemical reaction zone at higher dilutions.

TABLE VII.

Nucleo proteids treated with mineral acids.

Final Dilutions of Acids.	Hydrochloric.					Sulphuric.				
	Dilutions of Nucleo Proteids.					Dilutions of Nucleo Proteids.				
	1/2	1/4	1/8	1/16	1/32	1/2	1/4	1/8	1/16	1/32
Normal.....	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
1/10-1/100.	–	–	–	–	–	++	+	–	–	–
1/200......	+++	–	–	–	–	+++	+++	–	–	–
1/400......	–	+++	–	–	–	–	+++	+++	–	–
1/800......	–	–	+++	–	–	–	–	+++	+++	–
1/1500.....	–	–	–	+++	–	–	–	–	+++	+++
1/3000.....	–	–	–	–	+++	–	–	–	–	++

When nucleo proteids are treated with acetic or oxalic acids (Table VIII.), we do not find the same regularity in the flocculation zones as is observable with serum or its constituents when treated with these acids. The lower limits of the zones with acetic acid are sufficiently regular to indicate a chemical reaction, but it requires a great deal more acetic acid to redissolve the nucleo proteid precipitate than is necessary for redissolving a globulin precipitate, and indeed this property is made use of in practice for separating nucleo proteids from globulins, sufficient excess of acetic acid being added to hold the globulin in solution without affecting the precipitate of nucleo proteids.

TABLE VIII.

Nucleo proteids treated with organic acids.

Final Dilutions.	Acetic Acid.					Oxalic Acid.				
	Nucleo Proteid, Dilutions of "Orig."					Nucleo Proteid, Dilutions of "Orig."				
Acids.	1/2	1/4	1/8	1/16	1/32	1/2	1/4	1/8	1/16	1/32
Normal.....	–	–	–	–	–	–	–	–	–	–
1/10	+++	+++	–	–	–	–	–	–	–	–
1/100	+++	+++	+++	+++	++	++	–	–	–	–
1/200	+	+++	+++	+++	+++	++	+++	–	–	–
1/400	–	–	+++	+++	+++	–	+++	–	–	–
1/800	–	–	–	+++	+++	–	–	+++	+++	+++
1/1500	–	–	–	–	+++	–	–	–	–	+++
1/2500	–	–	–	–	–	–	–	–	–	–

In the foregoing experiments the tables show that the lower or chemical reaction zones of flocculation are single. Referring, for example, to Table VII. we find that nucleo proteids 1/8th are flocculated by sulphuric acid from 1/400 to 1/800, below which point there is no flocculation, nor is there any at higher concentrations of the acid until we reach the salting out zone at normal. We have chosen such simple examples as illustrations in order to make as clear as possible that the reaction appears to be chemical. But we have occasionally met with a much more complicated condition of affairs, and several zones of flocculation, more or less distinct from each other, have appeared, not only with serum but also with its constituents separately, and with nucleo proteids. We may refer to Table IX. as an example, but we shall not enter more fully at present into the question of these complicated reactions, as we have not yet been able to determine the conditions under which they appear. Since, however, each separate zone descends in regular proportions with dilutions of the protein, the supposition that the reaction is a chemical one is not invalidated, although it appears as if there must be a special chemical reaction at each point of precipitation.

We have so far given examples in which the globulins were used in the form of sodium salts, and have found precipitation to occur as if due to the reaction $\mathrm{Glob.\ OONa} + \mathrm{HCl} = \mathrm{Glob.\ OOH} + \mathrm{NaCl}$, the globulin being again soluble in excess of HCl. But the precipitation cannot be due solely to the turning out of the Na as the following experiment shows. Presumably on dialysis of the serum the globulin salt dissociates and the Na dialyzes out, leaving the insoluble globulin acid. If the dialyzed serum is now centrifugalized, sedimentation of the globulin is complete and the albumin fraction may be decanted off as a perfectly clear fluid. But the sedimented globulins can only partially be recovered by washing, for on mixing with distilled water and again centrifugalizing, the sedimentation is incomplete and in the wash water there remains a stable suspension of globulin which can be passed even through hard filter paper without appreciable loss of capacity.

Such a suspension we have treated with HCl (Table IX.), and found the usual precipitation zone following a law of definite proportions. In this table we also find, and give as an example, a second zone of flocculation such as we also find occasionally with serum and its constituents, as already mentioned.

At 1/2 dilution of the globulin suspension there is a zone at N/3000 of the acid, and another at N/25,000, each of these zones descending regularly with dilution of the suspension.

TABLE IX.

Globulins in suspension treated with hydrochloric acid.

	Dilutions of Acid.							
Dilution of Suspension.	Normal.	1/10 to 1/2000.	1/3000.	1/6000.	1/12,000.	1/25,000.	1/50,000.	1/100,000.
1/2	++	–	+++	–	–	+++	–	–
1/4	+	–	–	+++	–	–	+++	–
1/8	+	–	–	–	+++	–	–	+++

Experiments with dyes. — Although there can be little doubt that in the lower zones of flocculation of serum and its constituents by acids we are confronted with a chemical reaction, yet there is no means of knowing what is the actual strength of the protein solutions, so that we cannot judge if their normality at the optimum flocculation points corresponds to the normality of the acids. We thought it might be possible to obtain analogous results with certain colloidal anilin dyes, whose constitution is known.

Congo red (molecular weight, 698) is a sodium salt and when treated with acid affords a blue precipitate of the Congo red acid. The basic Nile blue chloride (molecular weight, 400 approximately) is blue, but on treatment with sodium hydroxide there is a red precipitate of Nile blue base.

Table IX. shows that although in the case of Congo red (used as an indicator) the change of color occurs in equimolecular proportions, yet the flocculation limits follow the salting out type. In other words, the Congo red acid is not insoluble until a certain strength of acidity is reached. With Nile blue and sodium hydroxide, not only does the flocculation follow the salting out type, but the change of color follows the same rule. Why the change of color does not occur in equimolecular proportions is difficult to understand, but it seems possible that the molecule of the Nile blue base is itself blue, but at a certain strength of alkali undergoes a polymeric condensation to a red form, the first step in aggregation and precipitation.

We have experimented on similar lines with several other dyes, but always with the same results. They are salted out, but do not precipitate in equimolecular proportions with the acid or alkali respectively. Although, therefore, we failed to show the point we wished, yet the results seem to be of sufficient interest to warrant brief mention here.

TABLE X.

Flocculation of dyestuffs by electrolytes of opposite sign.

	Congo Red Dilutions.						Nile Blue Dilutions.				
HCl, Final Dilutions.	N/1000	N/2000	N/4000	N/8000	N/12,000	NaOH, Final Dilutions.	N/1000	N/2000	N/4000	N/8000	N/12,000
Normal.... to N1/100.....	+++ +++	+++ +++	+++ +++	+++ +++	+++ +++	Normal... to N1/200....	+++ +++	+++ +++	+++ +++	+++ +++	+++ +++
" 1/200.....	—	—	—	—	—	1/300....	+++	+++	+++	—	+++
" 1/1000....	— cc	—	—	—	—	1/1000...	—	—	—	—	—
" 1/2000....	—	— cc	—	—	—	1/2000...	— cc	— cc	— cc	— cc	— cc
" 1/4000....	—	—	— cc	—	—	1/3000... to	—	—	—	—	—
" 1/8000....	—	—	—	— cc	—	1/∞.....	—	—	—	—	—
" 1/12,000..	—	—	—	—	— cc						

c. c. = Change of color. With Congo red the change is from red below the c. c. to blue at and above the c. c. The change occurs in equimolecular proportions except in column two, in which there is some variation, probably due to error of manipulation. The precipitation zone follows the salting out type from normal to N/100 throughout.

With Nile blue the change of color from blue below the c. c. to red at and above the c. c. does not occur in equimolecular proportions.

Before concluding this article we may return to the colloidal type of flocculation between two colloids of opposite sign worked out in Part I. and compare it with the chemical reaction type with acids just discussed.

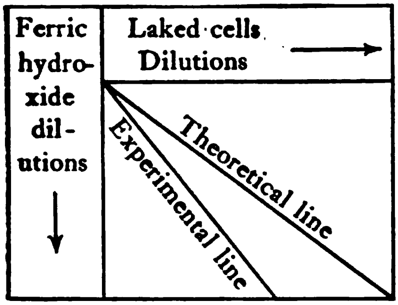
We have found in the chemical reaction type with acids that the flocculation zone strictly follows a law of definite proportions. As the amount of the protein sodium salt is reduced, to just the same extent is the amount of acid at the optimum point reduced. If on a diagram we draw a diagonal line representing the theoretical requirements for chemical reaction, we find such a line is strictly followed in actual experiment.

In the reaction between two colloids, *e.g.*, dyes, both of which are salts, the optimum flocculation zone strictly follows the theoretical line, as has already been pointed out in a previous paper.[5] The reaction is:

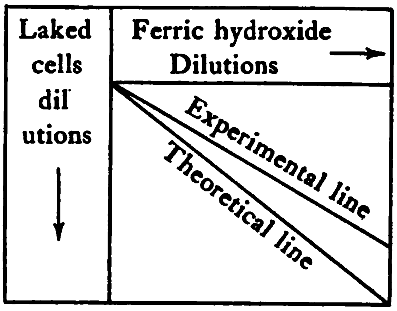
Dye acid Na + dye base Cl = NaCl + dye acid dye base, the dye acid dye base combination precipitating out.

If, on the other hand, we take the optimum points of flocculation between two colloids, neither of which, or only one of which is in the form of a salt, there can be no question of a chemical reaction between the ions of an electrolyte with formation of an inorganic salt such as NaCl. In such cases the theoretical line may be closely followed, but more usually it is not, and the experimental line falls on one side or the other of the theoretical line. With laked red cells and ferric hydroxide, for instance, we get lines as represented in the diagrams.

1



2



That is to say, a law of definite proportions is approximately followed but not at all closely. It seems possible that an explanation may be found if the reaction is regarded as one between aggregates and not between molecules. As the two colloids become diluted there is probably in most cases a rearrangement of the aggregates so that they tend to become smaller.

Let us assume that at the optimum point of flocculation between two colloids the number of aggregates of each colloid must be equal. Then we can picture two hypothetical cases:

A. — The aggregates of each colloid are rearranged "pari passu" on dilution.

Example: Each colloid at 1/10th "orig." 1,000 aggregates per ccm. = + + +.
" " at 1/1000th "orig." not 100 but 120 aggregates per ccm. = + + +.

The theoretical line would be closely followed.

B. — The aggregates of each colloid are rearranged unequally on dilution.

Example: Each colloid at 1/10th "orig." 1,000 aggregates per ccm. = + + +.
Positive colloid at 1/100th "orig." 120 aggregates per ccm. }
Negative colloid at 1/100th "orig." 110 " " " } = —.

The positive colloid would have to be a little weaker than 1/100th in order to bring the number of its aggregates down to 110 per ccm., and be flocculated by the negative colloid at 1/100th, so that in a table a law of definite proportions would appear to be violated. Nevertheless there may be such a law, but in this case it applies to aggregates and not to molecules. For such a reaction no constant could be found unless the relative sizes of the aggregates at each dilution could be determined.

The different types of flocculation of colloids including those dealt with in Part I. may be summarized as follows:

A. — Flocculation of colloids not in the form of salts.

1. Salting out type by electrolytes. Physical reaction.
2. Colloidal flocculation type. Reaction between aggregates. Probably physical reaction.
3. Irregular series type. A combination of these two types by salts of trivalent metals due to hydrolytic dissociation. The salting out zone is caused by the acid, and the colloidal flocculation zone by the hydroxide.

B. — Flocculation of proteins in the form of salts. — By acids. Irregular series.

a. A salting out zone at high concentration of the acid. Physical reaction.

b. A zone of flocculation varying with the strength of the protein according to a law of definite proportions. Chemical reaction.

C.—Two colloids of the opposite sign both in the form of salts (hydrochlorides and sodic as with dyes). There is a chemical reaction between the Cl and Na, and the two colloids precipitate in a combination which is probably of a physical nature.

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A STUDY OF SPECIFICITY AND SENSITIZATION BY MEANS OF THE CUTANEOUS TEST WITH HUMAN AND BOVINE TUBERCULIN.*

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Since November, 1908, I have performed the cutaneous test with tuberculin in the Medical Wards of the Massachusetts General Hospital on cases in which it was thought that it might be of service in diagnosis. In the course of this work some evidence on specificity and sensitization has accumulated.

I. SPECIFICITY.

(*a.*) In animals. — In a recent investigation[1] of the conjunctival tuberculin test in animals it was found that (1) non-tuberculous guinea-pigs fail to respond to the conjunctival test with old tuberculin ("T. O.") of the ordinary concentration or with stronger preparations in which the evaporation of the culture is carried to one-twentieth or even one-thirtieth of its volume; (2) positive conjunctival tests with old tuberculin of full strength may usually be obtained in guinea-pigs infected with human, bovine or avian tubercle bacilli at some period in the course of the disease, and when they occur appear to be specific for tuberculosis of the human, bovine and avian type; (3) the reaction, although specific for the group, appears not to be differential between the different types of infection. Animals infected with human, bovine or avian tubercle bacilli respond as well to the corresponding or different tuberculins. Unheated cultures of tubercle bacilli, concentrated in vacuo without heat and filtered through a porcelain filter, cause reactions

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* A continuation of the work undertaken under the Dalton Scholarship. From the Medical Wards of the Massachusetts General Hospital. Received for publication March 15, 1909.

in infected animals, but like old tuberculin are not differential in their action.

(*b.*) In clinical cases. — This "group reaction" with the various tuberculins after application to the conjunctiva of animals infected with the different types of tubercle bacilli suggests that a similar relation is likely to obtain in man. Slight degrees of difference in the intensity of the conjunctival reactions, however, are difficult of determination. Variations in the cutaneous response to tuberculin in man, on the other hand, are more easily observed and thus afford an opportunity for more accurate comparison of reactions following the application of different tuberculins. Variations in the relative intensity of cutaneous reactions obtained at the same time in the same individual with human and bovine tuberculin are often observed and have been thought to possess a specific and differential value, enabling the observer to conclude that the individual tested is the subject of infection with human or bovine tubercle bacilli or with one in greater proportion than the other. The problem could be directly solved only by the isolation and identification of the type of tubercle bacilli causing the reaction in each individual, but this is practically impossible. It may be indirectly approached by noting if the behavior of the reactions is such as to be consistent with specific differential properties in the different products. Thus a study of reactions following the performance of two tests at the same time, in the same individual, (1) with the same tuberculin, (2) with different tuberculins (human and bovine), and (3) by repetition, may be expected to afford some evidence in this matter.

Method. — In the application of the cutaneous tests the full strength of old tuberculin has been used. A fortunate abundance of material has permitted the use of the same preparations throughout, thus eliminating variations in the strength of different products as a source of error. For these tuberculins I am indebted to Prof. Theobald Smith. A uniform method of application has been adopted. The outer aspect of the upper arm has been prepared in the usual way. One drop of each of the tuberculins to be tested was placed on the arm and the underlying skin scarified by the rapid rotary motion of a platinum wire. The amount of force applied to the instrument was as nearly as possible the same for each scarification. The same instrument was used in all the tests. It consists merely of a heavy platinum wire (one and one-half millimeters in diameter) one end of which presents a flat surface, roughened by intersecting marks of a file, the other fixed to a handle of convenient size. The tuberculin was allowed to remain in contact with the scarified areas for ten minutes. The excess of fluid was then absorbed with sterile gauze. No bandage or other dressing has been applied. As a control, glycerine-bouillon culture material, prepared exactly as for the manufacture of tuberculin but without the bacilli, has been used. The inoculations were spaced at intervals of about four centimeters. They were observed after twenty-four and forty-eight hours and the diameter in millimeters of any resulting redness was then recorded. The comparative size of responses to the same or different tuberculins at the first application and after repetition was always noted after an equal interval of time. This interval was twenty-four hours, with rare exceptions. In the repetition of the test in the same individuals, the site of the first application was avoided. The outline of reactions following the inoculations was in general more or less circular. When this has not been the case, more than one measurement has been taken and the mean diameter recorded. The limits of redness, marking the transition from reacting areas to unaffected skin, were usually but not always well-defined. In rare instances, a faint blush faded imperceptibly into normal color. A survey of the figures obtained on measuring the extent of the controls, applied in every case, shows that they have practically never exceeded four millimeters in extent. A reaction of four millimeters or under with the tuberculin has therefore been considered negative; a response over four millimeters, as a positive test.

(1.) Comparison of two tests, simultaneously performed, with the same tuberculin, in the same individuals. — This was noted (Table I.) with human or bovine tuberculin in thirty-five instances. The two were unequal in twenty-four (68.5 per cent). The extremes of variation were 0.5 and 6.5 millimeters, with an average difference of 1.1 millimeters for the series.

The unequal responses in this group must be ascribed to a variation in the technic of the procedure. They are probably due to differences in the amount of force applied to the inoculating instrument.

TABLE I.

Comparison of two tests, simultaneously performed, in the same individuals, with the same tuberculin.

	Case No.	Extent Reaction in mm.		Difference.
		H. : H.	B. : B.	
1. Equal = 11.				
(*a*) Negative..	45	2.0– 2.0	....	0.0 mm.
	49	....	2.0 – 2.0	0.0 "
	96	....	2.0 – 2.0	0.0 "
	105	2.0– 2.0	....	0.0 "
	106	2.0– 2.0	....	0.0 "
(*b*) Positive...	42	....	7.0 – 7.0	0.0 "
	58	....	6.5 – 6.5	0.0 "
	97	....	4.5 – 4.5	0.0 "
	99	12.5–12.5	....	0.0 "
	103	7.5– 7.5	....	0.0 "
	109	....	10.0 –10.0	0.0 "

TABLE I. — *Continued.*

	Case No.	Extent Reaction in mm.		Difference.
		H. : H.	B. : B.	
2. Unequal = 24.				
(*a*) Negative..	57	....	2.5 - 3.0	0.5 mm.
	97	....	2.25- 3.5	1.25 "
	102	....	2.0 - 3.0	1.0 "
(*b*) One negative, one positive..	41	....	4.0 - 4.5	0.5 "
	96	....	3.0 - 4.5	1.5 "
	101	....	4.0 - 4.5	0.5 "
(*c*) Positive...	4	....	12.0 -12.5	0.5 "
	44	....	5.0 - 6.0	1.0 "
	46	8.0- 9.0	....	1.0 "
	47	10.0-13.0	....	3.0 "
	50	....	6.5 - 8.5	2.0 "
	59	....	5.5 - 6.0	0.5 "
	60	....	9.5 -11.5	2.0 "
	110	....	6.0 - 6.5	0.5 "
	95	....	6.5 - 7.5	1.0 "
	"	....	8.0 -10.0	2.0 "
	99	....	16.5 -20.0	3.5 "
	100	....	6.5 - 8.5	2.0 "
	"	....	13.0 -13.5	0.5 "
	101	....	8.5 -10.5	2.0 "
	102	....	10.0 -12.0	2.0 "
	103	....	10.5 -12.5	2.0 "
	104	9.5-16.0	....	6.5 "
	"	....	7.0 - 8.5	1.5 "
				35)38.75 mm.
Average difference..	....	....	....	1.10 mm.

Table II.

Comparison of two tests, simultaneously performed in the same individuals, with human and bovine serum.

	Case No.	H. - B.	Difference.
1. Equal = 25.			
(*a*) Negative (20)......	7	4.0- 4.0	0.0
	21	4.0- 4.0	0.0
	24	2.0- 2.0	0.0
	30	3.0- 3.0	0.0
	37	2.5- 2.5	0.0
	61	4.0- 4.0	0.0
	62	3.0- 3.0	0.0
	66	3.0- 3.0	0.0
	69	3.0- 3.0	0.0
	70	1.5- 1.5	0.0
	"	1.5- 1.5	0.0
	75	2.0- 2.0	0.0
	76	2.0- 2.0	0.0
	"	1.5- 1.5	0.0
	85	1.5- 1.5	0.0
	87	2.0- 2.0	0.0
	89	3.0- 3.0	0.0
	"	4.0- 4.0	0.0
	91	3.0- 3.0	0.0
	93	2.0- 2.0	0.0
(*b*) Positive (5)........	10	6.0- 6.0	0.0
	25	6.0- 6.0	0.0
	33	5.0- 5.0	0.0
	68	12.0-12.0	0.0
	83	6.0- 6.0	0.0

TABLE II.—*Continued.*

	Case No.	H.- B.	Difference.
2. Unequal = 79.			
(*a*) Negative (15)......	8	3.0- 4.0	1.0
	14	1.5- 2.0	0.5
	16	3.0- 2.0	1.0
	20	3.0- 2.0	1.0
	31	4.0- 3.0	1.0
	36	3.5- 3.0	0.5
	51	4.0- 3.0	1.0
	63	4.0- 3.0	1.0
	72	3.0- 4.0	1.0
	75	2.0- 1.5	0.5
	87	2.5- 3.0	0.5
	91	2.0- 4.0	2.0
	92	3.0- 3.5	0.5
	93	2.0- 2.5	0.5
	98	2.0- 3.0	1.0
(*b*) One negative, one positive (14).....	18	4.5- 3.5	1.0
	19	4.0- 4.5	0.5
	28	8.0- 3.0	5.0
	35	3.0- 6.5	3.5
	55	4.0- 7.5	3.5
	63	2.0- 6.0	4.0
	64	3.0- 6.0	3.0
	66	8.0- 3.0	5.0
	67	3.5- 5.0	1.5
	72	4.5- 4.0	0.5
	78	2.5- 6.5	4.0
	85	3.0- 6.5	3.5
	86	9.0- 2.5	6.5
	107	4.5- 4.0	0.5

TABLE II.— *Continued.*

	Case No.	H.- B.	Difference.
(*c*) Both positive (50) ..	5	8.0- 9.0	1.0
	6	20.0-15.0	5.0
	11	10.0- 8.5	1.5
	12	14.5-15.0	0.5
	13	7.0- 9.0	2.0
	15	6.0- 7.0	1.0
	17	14.0-13.0	1.0
	22	10.0-19.0	9.0
	26	20.0-12.0	8.0
	27	17.5-22.5	5.0
	29	8.0-11.0	3.0
	32	4.5- 6.5	2.0
	34	5.0- 7.5	2.5
	38	10.0-12.5	2.5
	39	11.0-12.0	1.0
	40	8.5- 9.5	1.0
	52	7.0- 7.5	0.5
	53	8.0- 9.5	1.5
	54	8.0- 9.0	1.0
	56	9.5-10.0	0.5
	61	7.0- 8.0	1.0
	62	7.0- 8.0	1.0
	64	7.0- 8.0	1.0
	65	8.0-17.5	9.5
	"	16.5-12.5	4.0
	71	9.5-14.5	5.0
	"	8.0-11.0	3.0
	73	7.0- 8.5	1.5

TABLE II. — *Concluded.*

	Case No.	H. - B.	Difference.
(*c*) Both positive (50) *continued*	73	12.0–11.0	1.0
	74	6.5– 8.5	2.0
	"	13.0–25.0	12.0
	78	6.5–12.0	5.5
	79	17.5–14.5	3.0
	"	18.5–20.0	1.5
	80	7.0–13.5	6.5
	"	9.0–11.5	2.5
	81	15.0–13.0	2.0
	"	13.5–12.5	1.0
	82	10.0–11.0	1.0
	"	12.5– 8.5	4.0
	83	13.0–15.5	2.5
	84	5.0–12.0	7.0
	86	8.0– 7.0	1.0
	90	7.5–10.0	2.5
	"	11.0–10.0	1.0
	92	7.0– 7.5	0.5
	94	10.5– 9.0	1.5
	"	11.5–14.5	3.0
	98	8.0– 9.0	1.0
	108	8.0– 7.5	0.5
			104) 193.0
Average difference	....	....	1.85 mm.

(2.) Comparison of two tests, simultaneously performed, with human and bovine tuberculin, in the same individuals. — This was investigated (Table II.) in one hundred and four trials. The two were unequal in seventy-nine (seventy-five per cent). The extremes of variation were 0.5 and 12.0 millimeters, with an average difference of 1.85 millimeters for the series.

If it be granted that human subjects are infected with human or bovine tubercle bacilli or with one in greater proportion than the other and if the cutaneous response to human and bovine tuberculin be capable of detecting the type of the infecting organism by the relative extent of such a response, it is then reasonable to assume that the comparison of results obtained with the same and different tuberculins would at least show a striking disparity. The proportion of unequal responses following the application of the same tuberculin (68.5 per cent in Table I.) and of human and bovine tuberculin (seventy-five per cent in Table II.) is not, however, strikingly different. The average difference in the extent of the response between two applications of the same product (1.1 millimeters in Table I.) is likewise only slightly less than the average difference with the different preparations (1.85 millimeters in Table II.). Moreover, if such slight differences as have been demonstrated with these different preparations are actually significant, they should be maintained in repetition. Their behavior in this respect was noted as follows:

(3.) Comparison by repetition. — Comparison of the results following the application of human and bovine tuberculin shows that the relation which obtained in the response at the first trial not infrequently fails to be maintained and may even be reversed in repetition. Thus of the one hundred and four trials (Table II.), twenty-eight are repetitions of the same tests, in the same individuals, after an interval of at least ten days. The relation which obtained in the first failed to be maintained in the second trial in sixteen, or more than half of the cases (Table III.).

Table III.

Comparison by repetition of two tests in the same individuals with human and bovine tuberculin.

	Case No.	1st Trial.	2d Trial.	
		H. : B.	H. : B.	
1. H. = B. and same on repetition .. 3	70	1.5– 1.5	1.5– 1.5	Same relation maintained in first and second trials.
	76	2.0– 2.0	1.5– 1.5	
	89	3.0– 3.0	4.0– 4.0	
2. H > B. and same on repetition .. 2	81	15.0–13.0	13.5–12.5	
	86	9.0– 2.5	8.0– 7.0	
3. H. < B. and same on repetition .. 7	64	3.0– 6.0	7.0– 8.0	
	71	9.5–14.5	8.0–11.0	
	74	6.5– 8.5	13.0–25.0	
	78	6.5–12.0	2.5– 6.5	
	80	7.0–13.5	9.0–11.5	
	92	3.0– 3.5	7.0– 7.5	
	98	2.0– 3.0	8.0– 9.0	
12				

TABLE III. — *Continued.*

	Case No.	1st Trial.	2d Trial.	
		H. : B.	H. : B.	
4. H. = B. on first, H < B. on second trial.......... 7	61	4.0– 4.0	7.0– 8.0	Relation between first and second trials not maintained or reversed.
	62	3.0– 3.0	7.0– 8.0	
	83	6.0– 6.b	13.0–15.5	
	85	1.5– 1.5	3 0– 6.5	
	87	2.0– 2.0	2.5– 3.0	
	91	3.0– 3.0	2.0– 4.0	
	93	2.0– 2.0	2.0– 2.5	
5. H. > B. on first, H. < B. on second trial.......... 1	94	10.5– 9.0	11.5–14.5	
6. H. > B. on first, H. = B. on second trial.......... 2	66	8.0– 3.0	3.0– 3.0	
	75	2.0– 1.5	2.0– 2.0	
7. H. < B. on first, H. > B. on second trial.......... 6	63	2.0– 6.0	4.0– 3.0	
	65	8.0–17.5	16.5–12.5	
	72	3.0– 4.0	4.5– 4.0	
	73	7.0– 8.5	12.0–11.0	
	82	10.0–11.0	12.5– 8.5	
	90	7.5–10.0	11.0–10.0	
16				

It seems reasonable to conclude, therefore, concerning this phase of specificity, that certain differences in the extent of the cutaneous response to tuberculin are to be expected. The difference in the extent of the response to human and bovine tuberculin in the same individual is not strikingly different from the response with two tests similarly performed with the same tuberculin and the relation may be reversed in repetition. The cutaneous tuberculin test in man thus fails to afford evidence of a specific and differential quality in human and bovine tuberculin. Its behavior in man is confirmatory of similar conclusions drawn from the study of differential tests in animals and still further emphasizes the practically identical relation, so far as reaction susceptibility is concerned, between the human and bovine types of tubercle bacilli.

While the results with human and bovine tuberculin in these investigations appear to correspond in man and in animals, I have noted an interesting difference in the

II. RESULTS WITH AVIAN TUBERCULIN.

(*a.*) In animals. — In the investigation of the conjunctival tuberculin test on animals infected with human, bovine or avian tubercle bacilli, it was found that avian tuberculin ("T. O.") of full strength (as well as human and bovine tuberculin) produced reactions in guinea-pigs infected with any one of the three types of tubercle bacilli. In general, however, the reactions were somewhat less intense and less constant with the avian than with the human or bovine tuberculin.

(*b.*) In clinical cases. — In forty instances the cutaneous test with avian tuberculin has been performed upon patients tested at the same time with human or bovine tuberculin and the control. The same preparation of avian tuberculin was used both in man and in animals. In thirty-nine of the tests the response from the application of the avian tuberculin has in general not exceeded that from the control — about two to three millimeters in diameter. In one instance only it reached five millimeters, but some transfer of material from a nearby area inoculated with bovine tuberculin could not be excluded. In twenty-nine of the forty tests the response to human or bovine tuberculin was simultaneously positive.

The explanation of this variation in reaction susceptibility to avian tuberculin between guinea-pigs and man is not clear.

and general appearance these colonies closely resemble a small meningococcus colony. Grown on the same sample of ascitic agar, the gonococcus colony is almost invariably smaller and more delicate than the majority of the meningococcus colonies. Exceptions to this rule, however, deprive this difference of its diagnostic value. As we have already indicated, certain meningococcus strains produce uniformly small colonies. On the other hand, two of our gonococcus strains grew luxuriantly when first isolated and presented appearances on ascitic agar plates identical with those usually assumed by the meningococcus. In comparing the growth of these organisms on the same medium, one difference was noticeable. While the meningococcus growth is viscid, it breaks up readily when transferred to a liquid menstruum. The gonococcus growth is somewhat more sticky, and when transferred to liquids tends to form shreds which do not break up readily. Such differences are, however, not sufficiently pronounced to be of any positive differential value.

Microscopic appearances. — The structure of the gonococcus colony bears a striking resemblance to a small meningococcus colony. They appear as small, faintly fawn-colored discs, with slightly irregular, fairly distinct outlines. The surface of the colony is covered with irregular, coarse crumbs, which are usually confined to the central portion and are smaller and less opaque than the corresponding structures in meningococcus colonies. The periphery of the colony is colorless and almost structureless. While fourteen gonococcus strains examined showed no crystalline deposits at any period of their growth, one strain was recently acquired which produced crystalline deposits in abundance after forty-eight hours' growth.

The earlier generations of the gonococcus grown on ascitic agar slants appear as discrete, gray, translucent colonies, usually smaller and more delicate than meningococcus colonies. Later generations produce a diffuse, gray, translucent growth which is, as a rule, less luxuriant than the meningococcus growth and is less easily removed from the surface of the medium. The water of condensation tends to remain clear. The surface of the fluid is covered with delicate flakes, and a mucinous, stringy material is found at the bottom of the fluid. A change in the color of the medium was never observed.

Cultural characters of the micrococcus catarrhalis. — Grown on ascitic agar plates this microörganism exhibits in the main two distinct types of growth.

Type 1. When the organism assumes this type of growth, the well isolated colonies appear as somewhat raised, opaque, grayish-white discs, measuring after twenty-four hours' incubation from one to two millimeters in diameter and presenting a slightly irregular moderately glistening surface. Examined with a reversed ocular the outlines are irregular, often having a jagged appearance resembling in this respect certain pseudodiphtheria colonies. Examined with a low power microscope, these colonies present numerous, irregular crumbs on the surface. They in which respect they differ from the meningococcus. In the course of our work we have encountered only two exceptions to this rule.

Cultural characters of the micrococcus pharyngis siccus. — Only two strains belonging to this group have been found in the course of our investigations. When first isolated these colonies are smaller than meningococcus colonies and can be readily differentiated from the latter by their consistence. They are very firm; it is almost impossible to break them up with a loop and, as a rule, they adhere closely to the medium. In normal salt solutions they sediment spontaneously. These characteristics suffice to distinguish them from the meningococcus. They resemble in some respects the micrococcus catarrhalis colony, but can readily be distinguished from this organism by means of fermentation tests as shown in the next section.

In subsequent transplants the organisms grow more luxuriantly. After twenty-four hours the colonies attain a size of from 2.5 to three millimeters and the surface is apt to become corrugated after the lapse of forty-eight or seventy-two hours.

Cultural characters of the diplococcus mucosus. — Grown on ascitic agar plates this organism develops colonies which resemble in many respects the growth of the meningococcus. After twenty-four hours' incubation they appear as gray or grayish-white, translucent discs measuring from three to four millimeters in diameter and presenting a smooth, glistening surface. They differ from the colonies produced by the meningococcus in that they are more succulent and mucinous in appearance, resembling in this respect the growth of the bacillus capsulatus mucosus group. They grow luxuriantly on serum free glucose agar and the growth tends to become confluent. The luxuriance of the growth on serum free media distinguishes it from that of the recently isolated meningococcus, which either fails to grow on such media or its development is slight. Moreover, this organism grows at room temperature, which further distinguishes it from the meningococcus. Finally, a film preparation prepared and stained according to the Hiss method reveals the presence of distinct capsules.

The differentiation of this organism from the meningococcus, therefore, presents no difficulties. It remains to be stated that microscopically the smallet colonies produced by the diplococcus mucosus closely resemble those formed by the meningococcus.

Cultural characters of the chromogenic Gram negative cocci. — The microörganisms included under this heading resemble each other very closely, morphologically, in their staining reactions and culturally. They differ, however, in their behavior towards carbohydrates. As we have already said, we have accepted these differences as a basis for classification, although in so doing we have included under one heading organisms which, according to their reactions with immune sera, do not belong to the closely resemble those of the micrococcus catarrhalis Type 1, and bear no resemblance to the growth of the meningococcus.

After more or less prolonged growth on artificial media, the converse is true. While the presence of a greenish-yellow pigment serves, as a rule, to distinguish this growth from that of the two species mentioned above, the amount of pigment produced is at times so slight as to render a differentiation difficult or impossible. We have in our collection a strain which temporarily lost its power to produce pigment. During this time the growth of the organism resembled the meningococcus in all respects. As we have already indicated, the presence of glucose favors pigment production, and thereby the recognition of this group is rendered less difficult.

Chromogenic group II. — The members of this group apparently belong to the same species. They all ferment dextrose, maltose, and levulose and respond to the action of the same immune serum, though not always to an extent which suffices to establish their identity. More convincing proof of their identity, viz., the demonstration of analogous absorptive properties, was not obtained, for reasons which will be given later. They correspond very closely, both culturally and biochemically, to the organisms described by von Lingelsheim[9] and named Diplococcus pharyngis flavus I.

Cultural characters. — Grown on glucose agar (one per cent glucose, one and one-half per cent agar — reaction + .8 per cent to phenolphthalein) at 36.5° C. After twenty-four hours' incubation, the well isolated colonies appear as slightly elevated, translucent discs, measuring from .7 to one millimeter in diameter. They are greenish gray by reflected light, greenish yellow by transmitted light and present a smooth glistening surface. The intensity of the color and the degree of opacity of the colonies produced by the different strains vary somewhat. Some of the members of this group produce colonies which are more opaque than meningococcus colonies, whereas the growth of others is about equally translucent. The growth is easily removed from the surface of the medium; its consistence closely resembles that of a meningococcus growth and when caught up in a loop the material has a distinct yellowish tint. In a fluid menstruum, some of the strains show a tendency to spontaneous sedimentation. A microscopic examination reveals greenish-fawn colored discs closely resembling small meningococcus colonies in structure. The surface of the colonies is covered with numerous rather coarse crumbs, chiefly confined to the central portion. The remainder of the colony is almost structureless. The margins are fairly distinct and only occasionally present slight irregularities.

After forty-eight hours' incubation the colonies measure from one to two millimeters in diameter, but in the main retain their original characters. Very often the central portions of the colonies are more opaque and more deeply tinted than the peripheral portions which may be almost transparent and wholly devoid of color. During the subsequent twenty-four hours the colonies continue to enlarge, reaching a size of from 2.5 to three millimeters. After seventy-two hours there is no further increase of the subject the reader may gain the impression that the recognition of the meningococcus in cultures is surrounded by very considerable difficulties. While it is true that a positive diagnosis of this organism cannot be made on morphological and cultural grounds, the identification of the meningococcus for practical purposes is relatively easy. The cultural characters of different meningococcus strains are remarkably uniform and the growth retains these characteristics even after prolonged cultivation on artificial media. If we confine ourselves to a consideration of recently isolated strains, we may say that it is hardly possible to confuse the meningococcus with the micrococcus catarrhalis or diplococcus pharyngis siccus. The same statement also applies to the members of the chromogenic groups I. and III. The chromogenic group II. may give rise to some diagnostic difficulties if the pigment production is slight, but this difficulty can be obviated by the use of glucose media for purposes of isolation, and if the precaution is adopted to view the growth by daylight. The diplococcus mucosus colonies, while they resemble the growth of the meningococcus in some respects, are usually more succulent and mucinous in character and, furthermore, films stained according to the Hiss capsule method suffice to differentiate them. The two remaining species, viz., the pseudomeningococcus and the gonococcus cannot be differentiated from the meningococcus either culturally or morphologically with any degree of certainty.

We may disregard the gonococcus for the moment, since other simple methods which will be mentioned in the next section suffice for the differentiation of this organism from the meningococcus. Concerning the identification and the differentiation of the pseudomeningococcus, which is the only other organism deserving serious consideration in this connection, we may say that while these organisms were set apart by us from the very beginning as a separate species owing to certain indefinable differences which a comparison of these strains with meningococcus strains revealed, it was sources. These precautions were fully justified by subsequent experiences.

Methods. — Culture media employed: Since the organisms in question were known to be rather exacting in the matter of nutriment, it was deemed advisable to employ a variety of media in the hope of finding one which is not only reliable but easily prepared.

Accordingly, the following media were used as a basis for these tests:

LIQUID MEDIA.

1. Nutrose water:
   Nutrose, one per cent; sodium chloride, .5 per cent; distilled water.
2. Nutrose peptone water:
   Nutrose, one per cent; Witte's peptone, one per cent; sodium chloride, .5 per cent; distilled water.
3. "Nährstoff Heyden" water:
   "Nährstoff Heyden," one per cent; sodium chloride, .5 per cent; distilled water.
4. "Nährstoff Heyden" peptone water:
   "Nährstoff Heyden," one per cent; Witte's peptone, one per cent; sodium chloride, .5 per cent; distilled water.
5. Beef serum water (Hiss):
   Beef serum, one part; distilled water, two parts.
6. Sheep serum water:
   Sheep serum, one part; distilled water, two parts.
7. Beef broth:
   This medium was prepared according to standard methods and rendered mildly alkaline to litmus.
8. Ascitic broth:
   Ascitic fluid, one part; sterile broth, two parts; final reaction of medium mildly alkaline to litmus.

Merck's highly sensitized litmus was used as an indicator. The amount of litmus required for the different media varied. The serum media received from one to 1.5 per cent of a five per cent watery solution of litmus, whereas .5 per cent to .75 per cent of the same solution sufficed for the perfectly transparent media.

The fermentable substances were added in one per cent proportion. To avoid the possible cleavage of sugars incident to prolonged sterilization, a ten per cent solution of the sugar in distilled water was prepared and sterilized at 100° C. for ten minutes and added to the medium which had been previously sterilized in bulk in the usual manner. The finished the growth, and in the case of the more or less turbid media, to determine the occurrence of growth.

II. Solid media. — All of the objections enumerated above are overcome by the use of solid media. We were led to their use by the work of von Lingelsheim,[9] who employed litmus agar media to which various sugars had been added. Instead of von Lingelsheim's plate method, however, we employed the agar slant method, which obviates several objections attending the use of plated media. In the tubed condition, contamination of the culture is less apt to occur and the period of observation can be extended over weeks. Very recently Shennan and Ritchie[27] suggested a similar modification of von Lingelsheim's method.

Toward the end of our investigation, we relied almost entirely upon this method. We are in the habit of preparing this medium in the following manner: five hundred grams of lean chopped beef are added to one liter of distilled water, thoroughly mixed and boiled over a free flame for fifteen minutes. During this time the mixture is stirred constantly. After filtering, an emulsion of Bacterium coli is added and the whole placed at incubator temperature for twenty-four hours. This mixture is now sterilized for ten minutes, filtered and 1 per cent peptone, .5 per cent sodium chloride and 1.6 per cent of agar agar previously soaked in water are added to the clear filtrate. A portion of the filtrate is placed in fermentation tubes and reinoculated with Bacterium coli to establish the sugar-free condition of the product. The mixture of agar and beef infusion is now placed in a water bath containing a saturated salt solution and boiled for forty-five minutes, suitable provision being made to prevent evaporation. It is now rendered mildly alkaline to litmus by means of a saturated solution of sodium carbonate. In correcting the reaction, due allowance is made for a slight augmentation of the acidity during the subsequent steps in the manufacture of this medium. It is now cooled to 55° C., and after adding the white of an egg it is steamed in the Arnold sterilizer until clarification of the upper strata of the medium has occurred. The clear supernatant fluid may now be pipetted off or the mixture may be filtered in the usual manner.

After transferring definite quantities to the required number of flasks the medium is sterilized for fifteen minutes on each of three consecutive days. Following the third sterilization, 1.5 per cent of a sterilized Kubel and Tiemann's litmus solution is added. One sample is set aside to be used as a control, whereas the others receive various carbohydrates (sterilized in solutions) in one per cent proportions.

The carbohydrate solutions were prepared in the following manner: To one hundred cubic centimeters of previously sterilized distilled water, we sugar media thus prepared has been insisted upon by many previous observers, a review of the literature shows that the method still enjoys a certain popularity.

The results obtained by us with media prepared in this manner were not consistent, and did not conform to those obtained with media prepared by the more complicated methods. Such conflicting results may be attributed to a cleavage of the carbohydrates resulting in the formation of variable amounts of certain derivatives capable of being acted upon by the microörganisms under examination. In the use of such media, therefore, we have no security that the results obtained are due to the action of the bacterium on the carbohydrate actually dissolved in the medium. While this, in itself, may not deprive the reaction of its diagnostic value, the fact that the extent to which a splitting of the sugars occurs probably varies in different samples of media may render the results obtained unreliable. Experimental proof supporting this view will be offered in the latter part of this section.

Aside from the possible sources of error arising from such alterations in the carbohydrate molecule, there are other objections to this method of preparing sugar media. Very often, faintly alkaline media containing carbohydrates become distinctly acid in the course of sterilization, rendering it difficult to recognize a subsequent slight augmentation of the acidity due to bacterial activities. Moreover, the change in reaction is not uniform in all the tubes made from the same sample of medium. The lack of uniformity in the appearance of different tubes may be ascribed to variations in the amount of alkali given off by different test-tubes in the course of sterilization.

Under these conditions, it is impossible to provide proper controls for slight changes occurring in the inoculated tubes. Finally, some of our results seem to indicate that the acid bodies formed exert a prejudicial influence on bacterial growth. However, further investigations are necessary to establish this point.

maltose were not identical. While decolorization of the dextrose nutrose peptone water occurred quite frequently, and partial coagulation of the medium was only rarely noted, the converse is true of the corresponding maltose medium. Furthermore, the amount of acid produced in the maltose tubes showing no coagulation frequently exceeded that found in the corresponding dextrose tubes. Approximately, one-third of the strains tested caused coagulation of the beef serum containing maltose, whereas only one-tenth of the strains coagulated the same medium containing dextrose. Decolorization of this medium was not observed.

While growth usually occurred in the dextrose and maltose media, failure to develop in the other sugar media was relatively frequent, clearly indicating the favorable influence which the former sugars have on the development of the meningococcus.

Quantitative determinations of the amount of acid formed by the meningococcus in the presence of dextrose and maltose were carried out in the following manner:

Beef broth, prepared according to standard methods, served as a basis for these experiments. The reaction of the broth was varied for different series of tests in order to determine what influence, if any, the initial reaction of the medium had on the fermentative capacities of the organisms under consideration. The sugars were added in one per cent proportion in the manner already indicated. A series of tubes, each containing ten cubic centimeters of broth, were simultaneously inoculated and exposed to incubator temperature for one week. To insure growth, each tube received a considerable amount of a twenty-four-hour slant agar culture. A set of plain broth tubes made from the same lot of medium was similarly treated. Several uninoculated tubes from each set were placed under the same conditions, and served as controls. Special devices to avoid evaporation of the medium were not employed, since in our opinion the use of the uninoculated control tubes made such precautions unnecessary.

The final titrations were carried out as follows: The tubes were exposed to live steam for ten minutes. Five cubic centimeters of culture fluid were removed from each tube, diluted with forty-five cubic centimeters of distilled water and boiled over the free flame for three minutes. After adding one cubic centimeter of an alcoholic solution of phenolphthalein, samples were titrated while hot with a n/10 sodium hydrate solution.

In order to determine the amount of alkali produced in neutral broth (+ .2) each sample first received a definite amount of n/10 hydrochloric acid solution and was then titrated against the corresponding alkali solution.

The following table (I.), which records the results of this series of tests, requires some explanation. The figures in the fourth, fifth, and sixth sections indicate the percentage of normal sodium hydroxide or normal hydrochloric acid solution required to reëstablish the initial reaction of the medium. Section three records the average reaction of three uninoculated tubes belonging to each set of media. These were exposed to the same conditions as were the inoculated tubes.

Series I. includes many strains which had been under cultivation for months. The oldest strain in the series had reached its forty-sixth generation while the youngest strain was in its seventh generation.

Series II. and III. represent forty-eight strains selected at random from those employed in the first series. All of these strains were eight months older than when first tested. The broth employed for Series II. and III. was made at the same time, but the reactions of the two lots differed for reasons already indicated.

TABLE NO. I.

Quantitative changes in reaction produced by the growth of the meningococcus in dextrose maltose, and plain broth.

Section 1	Section 2	Section 3	Section 4			Section 5			Section 6		
Series.	Number of Strains Tested.	Initial Reaction of Broth.	Production of Acid Dextrose Broth.			Production of Acid Maltose Broth.			Production of Alkali Plain Broth.		
			Extremes.		Average.	Extremes.		Average.	Extremes.		Average.
			Low.	High.		Low.	High.		Low.	High.	
I. . . .	75	+ 1.4%	0.1%	1.4%	0.834%	0.1%	1.4%	0.992%	0.05%	0.7%	0.26%
II. . .	48	+ 1.4%	0.4%	1.7%	0.852%	0.6%	1.6%	1.002%	0.1%	0.9%	0.43%
III. . .	48	+ 0.2%	0.5%	1.6%	0.914%	0.7%	1.8%	1.075%	0.05%	0.7%	0.235%

A review of the preceding table bears out many of the observations made in connection with the qualitative tests. The amount of acid produced varies considerably for different strains. In this connection it is important to note that a few strains produced only .1 per cent acid, an amount which might easily escape notice in a qualitative test unless the medium employed is neutral or only very mildly alkaline in reaction. Other strains produced as much as 1.4 per cent acid in the period specified. While in general it may be said that older cultures exhibit greater fermentative capacities than do younger cultures, there are many exceptions to this rule. For example, the youngest culture in the first series produced more acid in its action on dextrose and maltose than did the oldest strain. The former produced 1.2 per cent acid in dextrose broth and 1.3 per cent acid in maltose broth as compared to .5 per cent acid and 1.05 per cent produced by the oldest strain in the corresponding media. The variations in the capacity for growth exhibited by different strains regardless of their age may account for these differences.

In comparing the figures recorded in the second series with those of the first series, it will be noticed that a slight augmentation of the fermentative capacities has occurred during the eight months which elapsed between the first and second set of tests. While an examination of the original tables also supports this statement, it must be mentioned that the fermentative capacities of a few strains suffered slight impairment during the time specified.

The general average acid production is greater in maltose media than in dextrose media, a fact which was also observed in connection with the qualitative tests. In reviewing our original table, however, we find that while the majority produced more acid in maltose than in dextrose broth, the converse holds true for a few of the strains. The amount of alkali produced is also a variable factor which is in accord with the variations in the vital capacities of different strains suggested by other observations.

In comparing the second and third series it is further apparent that the initial reaction of the medium exerts no very pronounced influence on the fermentative capacities nor upon the growth of these organisms. The acid production on the whole is slightly greater in the broth reacting + .2 per cent to phenolphthalein, while the alkali production is less marked than in the corresponding media reacting + 1.4 per cent to phenolphthalein. These tests also show that the amount of acid tolerated by the meningococcus is very considerable, while it appears to be more sensitive to alkali. A few of the tubes showed an acidity of + 3.1 per cent, while — .5 per cent to phenolphthalein represents the greatest degree of alkalinity observed. The reasons for the differences observed in the broth media differing in their initial reactions may also be inferred from these facts. Owing to the great differences in the fermentative capacities exhibited by different meningococcus strains, little was to be expected from such determination in the way of help for purposes of differentiation. We have, however, undertaken similar quantitative tests with the other Gram negative cocci which need not be here recorded, since they failed to serve the purpose for which they were designed.

The other fermentable substances in the pure state were not affected by the meningococcus. The inoculated media containing these substances either remained unchanged in the course of one week, or showed a moderate grade of alkali production. The discordant results obtained with sugar media which had been subjected to fractional sterilization will be considered in detail toward the end of this section.

The action of the pseudomeningococcus on carbohydrates. — Six strains belonging to this group were tested on the various carbohydrates enumerated above. These strains failed to reveal either qualitative or quantitative differences in their behavior towards carbohydrates, which might serve to distinguish them from the meningococcus.

The action of the micrococcus catarrhalis on carbohydrates. — The micrococcus catarrhalis has no influence on any of the fermentable substances tested. The sugar media inoculated with this organism showed no appreciable change in reaction within twenty-four hours. In the course of one week distinct alkali production was manifest.

The action of the gonococcus on carbohydrates. — The fermentative capacities of the gonococcus grown on liquid media are slight, and the results obtained were not uniform owing to the fact that some strains failed to grow vigorously on such media.

Grown on solid media the gonococcus yielded uniform results, and the reactions, though slight, were usually apparent within thirty-six hours. After forty-eight hours' incubation, distinct acid production was noticeable in the dextrose tubes. The other sugar media remained unchanged, even after one week's growth, or showed slight alkali production. Twelve gonococcus strains tested on solid media fermented dextrose. Of eight strains tested in liquid media, only five produced acid in their action on dextrose and in all instances acid production was much delayed. An appreciable change in the media did not occur until forty-eight hours had elapsed. The amount of acid produced in liquid media was always slight, and no appreciable increase occurred during the succeeding days. The growth of the strains yielding negative results in dextrose media was not vigorous, whereas those yielding positive results showed a good growth.

The action of the micrococcus pharyngis siccus on carbohydrates. — While the organisms which we have grouped under this heading resemble very closely the organisms described and thus named by von Lingelsheim,[9] they differ in their fermentative properties. Our strains ferment glucose, maltose, levulose, and saccharose, whereas von Lingelsheim's did not ferment the last-named sugar. This difference may be due to the fact that his observations extended over a period of twenty-four hours only, whereas our observations on fermentation tests extended over a period of one week. We may say in passing that in our experience twenty-four hours is not always sufficient to establish the fermentative capacities of these organisms. In preparing culture media, it is very difficult to attain absolute neutrality of a mixture. A slight grade of alkalinity might be sufficient to neutralize the acid produced within twenty-four hours. Acid production may, therefore, not become apparent until later on in the experiment.

Shennan and Ritchie[27] describe two strains which closely resembled the von Lingelsheim organism but also fermented saccharose. They differed from those encountered by us in that they ferment galactose as well. Our experiences with this sugar, which will be considered presently, have made us rather sceptical towards positive reactions obtained by its use.

The action of the chromogenic Gram negative cocci on carbohydrates. — The members included in this group may be divided according to their action on carbohydrates into three sub-groups: I., dextrose, maltose, levulose, and saccharose fermentors; II., dextrose, maltose, and levulose fermentors; III., dextrose and maltose fermentors.

The following statement based on tests made with nutrose peptone water may be made concerning the action of this group of organisms on dextrose and maltose. While all the strains included in this series ferment dextrose and maltose, the amount of acid produced in the course of one week's growth is variable. As a rule, acid production becomes apparent in liquid media after twenty-four hours' incubation. In a few instances forty-eight hours elapsed before distinct acidity of the medium was manifest. Coagulation of the nutrose peptone water was not observed. Quantitative determinations of the acid formed after one week's growth revealed variations in the amount formed by different members belonging to the same sub-group, but the general average for the different sub-groups was approximately the same. Such determinations, therefore, furnish no data which might serve to differentiate the different sub-groups from each other and, as we have already indicated, they also fail to differentiate these organisms from the meningococcus.

Concerning the results obtained with the organisms (subgroup I.), which also ferment levulose and saccharose, the following statements obtain. While distinct turbidity of the inoculated levulose and saccharose media, indicating growth, is noticeable after twenty-four hours' incubation, there is no appreciable change in the reaction of the medium until forty-eight hours have elapsed. It would seem, therefore, that these substances are less easily fermented than are dextrose and maltose. The amount of acid formed by different strains is variable, but none of them formed a sufficient amount of acid to cause the medium to assume a milky character.

The members of the sub-group II. also ferment levulose but do not act on saccharose. Their capacity to ferment levulose is distinctly greater than that possessed by the preceding group. The change in reaction toward acidity is noticeable after twenty-four hours' incubation, and the medium becomes milky in character after five days have elapsed. Quantitative determinations also showed that the amount of acid formed by the members of this group in the presence of levulose exceeds that formed by the preceding group.

Fermentation tests with these organisms grown on solid media confirmed the results obtained with fluid media. While acid production by the members of group I. in levulose and saccharose was usually apparent within twenty-four hours, the change in the reaction was less pronounced than in the corresponding glucose and maltose media, and its appearance in tests made with some recently isolated strains was not infrequently delayed for thirty-six or forty-eight hours.

Before summing up our conclusions regarding the differential value of these tests, we may briefly review the literature pertaining to this phase of the subject and also attempt to account for the discordant results obtained by different observers.

Concerning the action of the meningococcus on glucose and maltose, perfect unanimity of opinion prevails. All observers, Dunham,[30] von Lingelshein Sholly,[24] Dunn and Gordon,[31] Kutsche mers and Wilson,[33] Arkwright,[34] and o both these substances are fermented b Differences of opinion, however, e behavior of the meningococcus towar and dextrin. Von Lingelsheim, Ku Wilson, and Shennan and Ritchie found when incorporated in solid media, are meningococcus. This has also been such media.

Diverse opinions concerning the fe carbohydrates by the meningococcus a who employed liquid media as a b According to the findings of Dunn a Mottram and Williams,[35] Andrewes,[32] meningococcus ferments galactose whe fluid medium. Shennan and Ritchie a meningococcus strains ferment this sug media were employed for the tests. T Arkwright[34] and Andrewes,[32] find that by this organism. The latter observer list of substances fermented by the men

Before discussing these results, it w introduce at this juncture a series of ex designed to determine the influence whi of sterilizing sugar media would have mentation tests.

In the following table (II.) we prese with levulose media which had been different means. Column I. contains with nutrose peptone water, which was to the finished and sterilized product levulose in distilled water. Column obtained with a medium identical in com sterile by the usual method of inte namely, exposure to a temperature of minutes on each of three consecutive the first instance had been exposed to th for ten minutes only, as compared with forty-five minutes' exposure in the second medium. Moreover, the sugar in the second instance was not only exposed to the influence of heat but also to that of a small amount of alkali, whereas in the first medium it was heated in a perfectly neutral menstruum.

TABLE No. II.

Showing the results of fermentation tests with levulose media sterilized by different methods.

		Sterilized 10 Minutes.				Sterilized 15 Minutes on 3 Consecutive Days.			
		24 hours.	48 hours.	72 hours.	1 week.	24 hours.	48 hours.	72 hours.	1 week.
Chromogenic Gram negative cocci.	L.Y. 1	O	O	※	+	※	+	+	+
	" 2	O	※	±	↔	※	+	+	+
	" 3	O	※	※	±	O	+	+	+
	" 4	O	±	±	↔	O	O	※	±
	" 5	O	※	±	↔	※	※	※	±
	" 6	O	※	※	+	O	※	±	+
	" 7	O	±	±	↔	O	※	※	+
	" 8	※	±	+	↔	※	+	+	↔
	" 9	±	+	+	↔	※	+	+	↔
	" 10	※	±	±	↔	※	+	+	↔
	" 11	±	±	+	↔	※	±	±	↔
	" 12	O	⊢	—	=	O	O	※	±
	" 13	O	O	—	=	O	O	※	±
	" 14	O	O	—	=	O	※	※	※
	" 15	⊢	⊢	—	=	O	O	O	※
	" 18	O	O	—	=	O	※	※	※
	" 22	⊢	⊢	—	=	O	O	±	±
Meningococci.	M. 250	—	—	—	=	※	±	※	=
	" 130	O	—	—	=	※	±	※	=
	" 101	O	—	—	=	※	※	※	=
	" 12	O	O	—	=	±	±	O	⊢
	" 155	O	O	—	=	±	±	±	⊢
	" 2451	O	O	—	—	※	±	±	⊢
	" 9	O	O	—	=	±	±	±	⊢

※ Very faintly acid.
± Faintly acid.
+ Frankly acid.
↔ Strongly acid.

⊢ Trace of alkali.
— Distinctly alkaline.
= Strongly alkaline.

If we compare the records tabulated above we find very striking differences in the results obtained. According to the first series of tests only certain strains ferment levulose, whereas in the second series all the strains produce acid, which in the absence of a control series would be attributed to their action on this sugar. We must infer, therefore, that although the ingredients employed in the preparation of both media were the same, the final composition of the two mixtures is not identical. Concerning the nature of the change which has occurred in the second sample the following observations furnish a clue: If a one per cent watery solution of levulose is rendered alkaline to a degree approximating that of nutrose peptone water and boiled over the free flame for a few minutes or exposed to live steam for ten minutes, a distinct change in the color as well as in the reaction occurs. The mixture is decidedly less alkaline and may now be neutral or faintly acid in reaction. It is clear, therefore, that some alteration has occurred in the process of sterilization.

According to Lobry de Bruyn and van Ekenstein,[37] small amounts of alkali and heat acting on levulose yield glucose. The presence of traces of glucose in the medium exposed to fractional sterilization would explain the reactions obtained with this medium. The lack of constancy in the reactions obtained with this sugar is apparently determined in part by the extent of the transformation which it has undergone in different samples of media, and in part by the amount of alkali produced by the organism tested. A review of the results obtained with meningococcus strains shows that the amount of fermentable material formed in a medium exposed to intermittent sterilization may be inconsiderable. The amount of alkali produced by these strains in the course of one week was sufficient to completely neutralize the small amount of acid formed during the first three days. These experiments have been repeated and similar results obtained.

The following table (III.) is inserted to establish the same facts for galactose media improperly prepared:

TABLE No. III.

Showing the results of fermentation tests with galactose media sterilized by different methods.

		Sterilized 10 Minutes.				Sterilized 15 Minutes on 3 Consecutive Days.			
		24 hours.	48 hours.	72 hours.	1 week.	24 hours.	48 hours.	72 hours.	1 week.
Chromogenic Gram negative cocci.	L.Y. 1	O	O	—	=	O	±	±	±
	" 2	O	O	—	=	※	±	±	±
	" 3	O	O	—	=	※	±	±	±
	" 4	O	O	—	=	※	O	O	O
	" 5	O	O	—	=	※	±	※	O
	" 6	O	O	—	=	O	O	O	※
	" 7	O	O	—	=	O	※	※	※
	" 8	—	—	—	=	※	※	±	※
	" 9	⊢	⊢	—	=	O	O	⊢	⊢
	" 10	—	—	—	=	※	±	±	±
	" 11	⊢	⊢	—	=	O	※	※	O
	" 12	O	O	—	=	O	O	⊢	—
	" 13	O	O	—	=	※	※	※	—
	" 14	O	O	—	=	O	O	O	—
	" 15	O	O	—	=	※	※	O	O
	" 16	O	O	—	=	※	※	O	O
	" 18	O	O	—	=	O	O	O	O
	" 22	⊢	—	—	=	O	O	O	O
Meningococci.	M. 250	O	⊢	—	=	±	±	±	±
	" 12	O	—	—	=	±	±	±	±
	" 101	O	O	—	=	±	±	±	±
	" 155	O	O	—	=	±	±	±	±

※ Very faintly acid.	⊢ Trace of alkali.
± Faintly acid.	— Distinctly alkaline.
+ Frankly acid.	= Strongly alkaline.

That this sugar is profoundly altered by heat is shown by a very distinct reduction in the alkalescence of a galactose medium exposed to intermittent sterilization. According to Lobry de Bruyn and van Ekenstein,[37] galactose heated in the presence of an alkali is converted into a number of isomeric sugars.

The following tests were undertaken to determine the influence of sterilization on galactose in a neutral and in an alkaline mixture, and to determine, moreover, the occurrence, if any, of further alterations at incubator temperature.

Experiment. — A two per cent galactose solution in distilled water was prepared. Every means was adopted to obtain the solution free from all traces of alkali. The water employed was freshly distilled. All the vessels and pipettes employed for the preparation of the various mixtures had been previously steamed and consisted of Jena glass, which is known to give off very little alkali. The galactose solution received .5 per cent of a five per cent watery (distilled) solution of Merck's highly sensitized litmus and the whole was divided into three parts.

Sample No. I. = Neutral in reaction.

Sample No. II. = Received .025 cubic centimeter n/10 sodium hydrate solution per ten cubic centimeters — approximate percentage of alkali present = .001 per cent.

Sample No. III. = Received .05 cubic centimeter of n/10 sodium hydrate solution per ten cubic centimeters — approximate percentage of alkali present = .002 per cent.

These samples were further subdivided, transferred to special tubes made of so-called alkali free glass, and treated as indicated in the following table. It remains to be stated that all the tubes were sealed with paraffin to avoid evaporation.

TABLE NO. IV.

Showing the effect of heat on galactose dissolved in a neutral and in an alkaline menstruum.

Readings.	Reaction Neutral.				0.001% Alkaline.			0.002% Alkaline.		
	Unheated.		Heated 100° C. 20 minutes.		Un-heated.	Heated 100° C. 20 minutes.		Un-heated.	Heated 100° C. 20 minutes.	
	Sample I. Kept at 6°–8° C.	Sample II. Kept at 36½° C.	Sample III. Kept at 6°–8° C.	Sample IV. Kept at 36½° C.	Sample V. Kept at 6°–8° C.	Sample VI. Kept at 6°–8° C.	Sample VII. Kept at 36½° C.	Sample VIII. Kept at 6°–8° C.	Sample IX. Kept at 6°–8° C.	Sample X. Kept at 36½° C.
Immediate. Following sterilization of certain samples.	Reaction unchanged.	Reaction unchanged.	Barely perceptible change towards acidity.	Same as Sample III.	Reaction unchanged.	Compared with Sample V. shows distinct acid formation.	Same as Sample VI.	Reaction unchanged.	Distinct acid formation. Reaction now approximates that of Sample I.	Same as Sample IX.
After one week.	Reaction unchanged.	Reaction unchanged.	Same as first reading.	Compared with Sample III. perhaps a trifle more acid.	Reaction unchanged.	Same as first reading.	Compared with Sample VI. distinctly more acid. Compared with Sample I. is frankly acid.	Reaction unchanged.	Same as first reading.	Compared with Sample IX. distinctly more acid. Compared with Sample I. is frankly acid.

In reviewing the results of this experiment, the following conclusions seem justified:

1st. That galactose dissolved in alkali free (?) distilled water and heated for twenty minutes at 100° C. undergoes practically no change which is demonstrable by the indicator employed and that the changes in reaction occurring during subsequent incubation extending over a period of one week are so slight as to be negligible.

2d. That galactose dissolved in distilled water containing

TABLE No. V.

Showing results of fermentation tests with media containing impure dextrin.

		24 Hours.	48 Hours.	72 Hours.	1 Week.
Meningococci.	M. 1	±	±	±	⊢
	" 2	±	±	±	⊢
	" 3	±	±	±	⊢
	" 9	+	+	±	⊢
	" 24	±	±	±	⊢
	" 88	±	±	±	—
	" 71	±	±	±	—
Chromogenics.	L.Y. 1 I.	±	±	±	—
	" 2 I.	±	±	±	—
	" 8 II.	O	±	±	O
	" 9 II.	O	±	±	O
	" 12 III.	±	±	±	⊢
	" 13 III.	O	±	±	±
Micrococcus catarrhalis.	M.C. 55	O	O	O	—
	" 56	O	O	⊢	—

± Faintly acid. ⊢ Trace alkali. — Distinctly alkaline.

A review of these results reveals the fact that the meningococcus and allied Gram negative cocci produce only a slight grade of acidity in dextrin during the first twenty-four hours, which does not increase during the second twenty-four hours and subsequently gives way to a faint alkaline reaction. These peculiar results we have attributed to the presence of other substances capable of being fermented by these organisms. The prompt though slight change in the reaction of this medium indicates that it contains substances which are easily fermented. The fact that there are no further changes towards acidity at the subsequent readings shows that all of the available fermentable material is exhausted early in the course of the test. That the organism does not cease to grow is indicated by the alkali production which follows the initial acid production. These facts are incompatible with the idea that the organisms ferment dextrin. Very recently we succeeded in obtaining a sample of dextrin which was apparently free from such impurities. Negative results were obtained with this product.

Concerning the nature of the impurities assumed to be present in dextrin, Maquenne's[38] observations furnish a clue. This observer finds that so-called chemically pure dextrin contains maltose or isomaltose or glucose in small quantities. The presence of traces of glucose or maltose would fully explain the reactions obtained by us.

It seems probable, therefore, that the positive results obtained by a number of observers with levulose and galactose may be attributed to the method of sterilization employed by them, which led to a cleavage of these substances. In every instance these sugars were exposed to the action of heat in the presence of alkali and the other components of the medium. The degree of heat employed for the sterilization of the liquid media is not indicated in the records excepting in the report of Rundle, Moltram and Williams.[35] These observers boiled an alkaline galactose medium for twenty minutes and steamed the same for twenty minutes on three consecutive days. The amount of heat employed in this instance is ample to profoundly alter these sugars.

The positive results obtained with dextrin medium by one observer we are inclined to attribute to the presence of impurities in the products employed. The fact that tests with solid media furnished no discordant results also appears to be due to the care taken in the preparation of such media to prevent a cleavage of the sugars. Von Lingelsheim[9] and ourselves sterilized the sugars separately and employed distilled water as a solvent. Shennan and Ritchie[27] added the sugars to the unneutralized agar and subsequently sterilized the same for fifteen minutes on two occasions. While the amount of heat employed in this instance was considerable, it was apparently not sufficient to cause in this menstruum a cleavage of the carbohydrates.

Concerning the fermentative properties of the gonococcus, the literature contains the following references: Dunn and Gordon,[31] Arkwright,[34] and Shennan and Ritchie[27] find that this organism ferments glucose and galactose. While we are able to confirm the observations concerning glucose, we cannot accept the observations made on galactose media. In the unaltered state galactose was not fermented by the fifteen gonococcus strains tested by us.

According to Wollstein[39] the gonococcus ferments glucose and maltose. We have repeatedly tested gonococcus strains in maltose media, both liquid and solid, and have always obtained negative results. Wollstein's paper contains no intimation concerning the methods used for the sterilization of the sugar media. Impurities in the maltose used or the presence of cleavage products due to prolonged sterilization of the medium may explain the anomalous results obtained by this observer. Very recently Rothe[40] reported a series of fermentation tests undertaken with gonococcus strains. Dextrose, maltose, and levulose, incorporated in solid media prepared according to von Lingelsheim's method, were employed by this observer. According to these investigations, the gonococcus ferments only glucose.

With the exception of Celler[41] general agreement prevails concerning the behavior of the micrococcus catarrhalis towards carbohydrates. This organism fails to ferment any of the carbohydrates usually employed for the differentiation of bacteria. Celler finds that the micrococcus catarrhalis ferments glucose and saccharose. It seems likely that the strains yielding these results belonged to some other species, possibly to members of our chromogenic group I.

Concerning the behavior of the chromogenic group towards carbohydrates, very little need here be said since the identity of the organisms studied by different observers has not been established.

For the sake of completeness, however, we may briefly record the work of von Lingelsheim[9] concerning Gram negative chromogenic cocci. This observer encountered three different species which he named Diplococcus Flavus I., II., III. The first two species, while they ferment the same carbohydrates, namely, glucose, maltose, and levulose, differ somewhat culturally and are easily distinguished by means of agglutination tests. It is probable that the diplococcus flavus I. corresponds to the members of our chromogenic group II. The diplococcus flavus II. we have either not encountered or we have failed to recognize them as a distinct species and have grouped them with our chromogenic group II.

Some of the recently isolated strains included in group I. failed to produce acid in saccharose media within twenty-four hours. Since von Lingelsheim's observations did not extend over a longer period it is also possible that the organisms designated as diplococcus flavus II. belong to our chromogenic group I.

Concerning the diplococcus flavus III., we may say that we isolated four strains which corresponded culturally and biochemically to the description of this species given by von Lingelsheim. These strains were, however, short lived. All of them died out before our observations were complete. For this reason we have omitted them from this report.

In conclusion we may summarize our results in the following Table VI. In view of the experimental evidence, which has been recorded in the preceding pages, we feel justified in assuming that the following records indicate the actual fermentative capacities of the organisms under consideration.

TABLE NO. VI.

Summary of fermentation tests with Gram negative cocci.

Number of Strains Tested.		Dextrose.	Maltose.	Levulose.	Saccharose.	Lactose.	Galactose.	Dextrin.	Mannite.	Dulcite.	Inulin.
Meningococcus,	**200 strains.**	+	+	o	o	o	o	o	o	o	o
Pseudomeningococcus,	6 "	+	+	o	o	o	o	o	o	o	o
Gonococcus,	15 "	+	o	o	o	o	o	o	o	o	o
Micrococcus catarrhalis,	64 "	o	o	o	o	o	o	o	o	o	o
Micrococcus pharyngis siccus,	2 "	+	+	+	+	o	o	o	o	o	o
Chromogenic group I.,	28 "	+	+	+	+	o	o	o	o	o	o
" " **II.,**	11 "	+	+	+	o	o	o	o	o	o	o
" " **III.,**	9 "	+	+	o	o	o	o	o	o	o	o
Jaeger meningococcus, Král,	1 "	+	+	+	+	+	+	o	o	o	o
Diplococcus crassus, Král,	1 "	+	+	+	+	+	+	o	o	o	o

The diagnostic value of fermentation tests with the Gram negative cocci may be inferred from these results. We are able by these means to differentiate the meningococcus from the gonococcus, micrococcus catarrhalis, diplococcus pharyngis siccus and from the chromogenic cocci included in sub-groups I. and II. The chromogenic sub-group III. and the pseudomeningococcus cannot be differentiated from the meningococcus by means of fermentation tests.

We have introduced in this table the results of fermentation tests with the so-called Jaeger type of the meningococcus and with the diplococcus crassus. These organisms ferment dextrose, maltose, levulose, saccharose, lactose, and galactose, which is in accord with the results obtained by von Lingelsheim.[9]

Summary. — From the results thus far recorded it is evident that a study of the cultural characters and biochemical properties of the Gram negative cocci suffices for the differentiation of these organisms. There is only one exception to this rule, namely, the pseudomeningococcus. For the differentiation of this organism from the meningococcus, serum reactions must be employed.

General summary and conclusions concerning the morphological, cultural, and biochemical proportions of the meningococcus and allied Gram negative cocci:

1. For the isolation of the microörganisms under consideration a mixture of ascitic fluid or human blood serum and agar or beef broth proved the most reliable culture medium.

2. The addition of glucose to the culture medium favors the growth of these organisms, and owing to the favorable influence which the sugar exerts on pigment production, its use renders the recognition of the chromogenic Gram negative cocci less difficult.

3. For the isolation of the meningococcus from the blood we have employed liquid media. For this purpose we recommend the use of considerable quantities of medium (one hundred cubic centimeters) contained in large flasks to

allow of free access of air. This suggestion is based on the fact that the meningococcus is an obligative aërobe.

4. For the isolation of the pseudomeningococcus strains we employed a mixture of human blood serum and agar. These organisms when first isolated failed to grow or showed only a feeble growth on ascitic agar.

5. Many of the Gram negative cocci failed to grow on serum free media when first isolated. After repeated transplantation the majority of the strains including the pseudomeningococcus and the gonococcus grew well on plain agar media, and better still on agar media prepared according to Thalmann's directions. The addition of glucose to this medium also proved advantageous.

6. For the maintenance of these organisms on culture media it is necessary to transplant recently isolated strains daily. After several generations the intervals for transplantation may be prolonged, and after several weeks' growth on artificial culture media, transplantation once a month suffices to keep most of the strains alive.

Certain meningococcus strains cannot be carried beyond the first or second generation, others thrive on culture media for many generations and then suddenly fail to develop when transplanted.

7. A morphological study of these organisms furnished no reliable evidence for purposes of differentiation. Slight differences in size and shape were observed, but these were not sufficiently constant to be of diagnostic value. The same may be said of the grouping of these organisms. The arrangement in pairs is on the whole more common than the grouping in tetrads. Chain formation was never observed.

8. All of the Gram negative cocci studied showed a more or less pronounced tendency to degenerate early in culture media. The time of appearance of involution forms, and the extent of such changes, is determined in part by inherent peculiarites of individual strains and in part by the composition of the culture medium.

9. In exudates, all of these organisms, in so far as we were

able to determine this point, exhibit a predilection for the interior of leucocytes.

10. Treated according to Gram's method they promptly lose the dye. Many of our meningococcus strains have been passed through the animal body repeatedly and have been maintained on culture media for long periods without suffering any alteration in their behavior towards the Gram method of staining. It may be accepted, therefore, that an organism which retains the dye when treated in this manner is not the meningococcus of Weichselbaum.

11. The Neisser method of staining serves to differentiate the gonococcus in cultures from the other Gram negative cocci. We are, however, not inclined to attribute to the method any positive differential value, since, according to one observer, the gonococcus in exudates also shows metachromatic structures.

12. The capsule stains serve to differentiate the diplococcus mucosus from the other Gram negative cocci studied. The diplococcus mucosus is the only encapsulated Gram negative coccus encountered by us in the course of this investigation.

13. The colony features of different meningococcus strains are remarkably uniform and constant. After prolonged cultivation these organisms grow more luxuriantly, but otherwise retain their original cultural peculiarities.

On ascitic agar plates the growth of the meningococcus is sufficiently characteristic to distinguish it from that of the micrococcus catarrhalis, of the micrococcus pharyngis siccus, and of the chromogenic Gram negative cocci. Difficulties may arise in connection with the members of chromogenic group II. when sugar free media are employed for isolation. Under these conditions the latter organisms may fail to produce pigment. The differentiation of the meningococcus from the pseudomeningococcus and the gonococcus cannot be made with certainty by an examination of the growth on ascitic agar plates.

14. While the meningococcus, the pseudomeningococcus, and the gonococcus retain their original cultural features

during prolonged cultivation, the cultural characters of the micrococcus catarrhalis and of the chromogenic groups I. and III. undergo marked changes in the course of time. After a variable period the growth of these organisms assumes many of the characteristics of a meningococcus growth.

15. Fermentation tests furnish reliable data for the differentiation of the Gram negative cocci. For diagnostic purposes we can recommend the use of solid sugar media, first introduced by von Lingelsheim. The reactions in solid media are usually manifest within twenty-four hours, while in liquid media the changes in reaction may be delayed until forty-eight hours or seventy-two hours have elapsed.

In the preparation of such carbohydrate media the usual method of intermittent sterilization should be avoided. The cleavage of the carbohydrate molecule occurring in the course of such sterilization deprives the results of their diagnostic value. Among the carbohydrates tested, levulose and galactose appeared to be particularly unstable, a fact which probably accounts for the discordant results obtained by different observers working with media containing these sugars.

16. Quantitative determinations of the amount of acid formed by the different Gram negative cocci in the presence of carbohydrates furnished no data which were available for purposes of differentiation.

17. The fermentative capacities of the meningococcus were not altered materially by prolonged growth on artificial media.

18. The so-called meningococcus of Jaeger represents a species quite distinct from the meningococcus. Morphologically, culturally, and biochemically these organisms do not resemble each other.

NOTE. — A description of the cultural peculiarities of the Jaeger coccus has been omitted, since the growth of the single strain which we have examined bears only a remote resemblance to that of the meningococcus. In size and general appearance the colonies of the Jaeger coccus resemble those of a streptococcus.

SERUM REACTIONS.

Albrecht and Ghon[6] in 1901 first called attention to the fact that the blood serum of animals receiving repeated injections of meningococci contained substances capable of clumping these organisms under suitable conditions. A systematic investigation of this subject was first undertaken by Jaeger.[42] The organisms employed by him corresponded in part to the genuine Weichselbaum diplococcus, and in part to the so-called Jaeger type of the meningococcus. The sera were all derived from rabbits. The main question at issue was the relationship of these two types to each other, and since both types responded to immune sera produced by members of either group, Jaeger assumed that the two types belonged to the same species. Sorgente[12] also employed rabbits and confirmed Jaeger's findings in so far as his investigations were parallel. The results obtained by Jaeger and Sorgente are, however, at variance with those obtained by later observers. Bettencourt and Franca[7] undertook parallel investigations, using horses, however, for the production of the immune sera. They were unable to establish any relationship between the two types of organisms referred to. Von Lingelsheim[9] repeated the work of Jaeger and found that whilst sera produced in rabbits by the injection of the diplococcus crassus, which according to this observer is identical with the Jaeger type of the meningococcus, failed to influence the Weichselbaum type when the serum was employed in high dilution, the sera produced in rabbits by injection of the latter type exercised a pronounced influence on the former. While von Lingelsheim offered no explanation for the peculiar results obtained with meningococcus immune sera acting on the diplococcus crassus and diplococcus Jaeger, he was not inclined to accept these reactions as indicating the identity of the two types.

Finally, Jochmann[43, 44] tested a series of strains representing both types and was unable to establish any relationship between these types by employing immune sera derived from horses.

In reviewing this work one fact stands out prominently, namely, that difficulties in the differentiation of these two types only occurred when rabbits' sera were employed. The significance of this fact will be considered in the section devoted to animal experimentation with the meningococcus.

The work of other investigators bearing on the subject of serum reactions will be considered as occasion arises for a comparison of their results with our own.

AGGLUTINATION TESTS.

Method. — We relied entirely upon the so-called macroscopic method for the determination of these reactions. Preliminary tests in which the microscopic method was tried yielded less satisfactory results. The standard suspensions employed throughout were prepared with filtered and sterilized .85 per cent salt solutions. Each cubic centimeter of salt solution received .004 gram of moist bacteria, and was shaken up thoroughly so as to form a perfect suspension. The same standard loop was employed throughout for measuring the bacteria in preparing the standard suspensions. To secure wholly comparable results the tests were carried out and the results recorded throughout by the same individual.

Various dilutions of the serum, graded in the customary manner, were prepared. Each tube in the series received the same amount of diluted serum, to which an equal amount of the standard suspension was added in a manner to insure a prompt mixing of the two fluids.

The same suspension diluted with an equal amount of .85 per cent salt solution served as a control. The series thus prepared was placed at incubator temperature for two hours and transferred to the ice-chest (9° C.) and kept at this temperature for twenty-two hours. Readings were usually taken after one, two, three, and four hours had elapsed, and the final control readings were made after the lapse of twenty-four hours. Particular attention was paid to the course of the reaction. It occasionally happened that no appreciable change in the appearance of the mixtures was noticeable after several hours had elapsed, whereas, after twenty-four hours, complete clarification of the fluids was noted, simulating a positive agglutination reaction in all dilutions. Whenever distinct progression in the reaction, both as to time and dilution, was not apparent the tests were repeated, and we were not surprised to find quite different results. While a partial sedimentation in the control suspensions occasionally attracted our attention to the spurious nature of these reactions, such a sedimentation in the control tubes did not occur invariably. These experiences lead us to conclude that the normal salt solution control which serves its purpose when less erratic organisms are being tested, is not a wholly reliable control in the case of the organisms under consideration. The tests here recorded were therefore controlled by the use of simultaneous tests with normal serum.

In passing, we may mention that very recently Eberle[46] recommended centrifugalization of the suspensions at a reduced rate of speed. This caused a sedimentation of all coarser particles suspended in the fluid and, according to this observer, prevented the occurrence of pseudoagglutinations.

The mixture showing the end point of the reaction, adopted by us as the standard, presented the following picture: The vast majority of the cocci were clumped and found at the bottom of the tube. The supernatant fluid presented a degree of turbidity which was just appreciable when viewed at an angle and by a good light. We have recorded such a reaction as positive. Partial reactions were not utilized in comparing the effects produced on different strains by the same serum for the following reasons: While at times a sharp distinction could be drawn between our end point and the appearances in the succeeding dilutions, it very frequently happened that partial reactions showing no appreciable differences were noted in several dilutions above this point. These peculiar results suggest that the individual organisms present in the same culture are not equally sensitive to the influence of specific agglutinins. When the end point of the reaction is sharply defined or when a gradual but progressive decline in the effects of the serum is observed in dilutions above this point, we may assume that all the individuals enjoy the same degree of agglutinability, whereas numerous partial reactions in a series might be explained by the presence of very agglutinable individuals intermingled with others that are less agglutinable.

Concerning the length of time required for the completion of the reaction, opinions differ. Most observers are agreed that the macroscopic test requires, at least, twenty-four hours. Dunham[30] and Eberle[46] found that the maximum reaction was not reached until forty-eight hours had elapsed. The results of our own experiments bearing on this point also indicate that forty-eight hours are necessary in some instances to complete the reaction, but in the main the values obtained were not materially altered by postponing the final readings to this extent. As a matter of convenience, therefore, we adhered to the twenty-four-hour limit.

The sera employed for these tests were derived from rabbits which were immunized by repeated intravenous injections of the organisms in question. As a rule the animals received weekly injections of bacteria suspended in normal salt solution, killed by exposure to a temperature of 65° C. for thirty minutes. We began by injecting .002 gram of moist bacteria and gradually increased the dose to .008 gram. While the immunizing procedure indicated above was generally followed, no effort was made to adhere to this rule. The dose, as well as the time of administration, was wholly determined by the general condition and weight of the animal after the preceding injection. The effects of such injections

will be considered in connection with the experiments designed to determine the pathogenicity of the organisms under consideration. Immunization with living cultures or with organisms killed by simple desiccation yielded less satisfactory results.

Agglutination tests. Meningococcus immune sera versus meningococcus strains. — One of the great difficulties encountered in connection with this work is the very pronounced instability of the agglutinable properties of the meningococcus. The agglutination tests made with the same strains and with the same serum on different days failed to yield uniform results, although the organisms were grown on the same lot of medium and under practically identical conditions. These variations were not due to alterations of the serum, but to an impairment or augmentation of the agglutinable properties of the strains occurring in the course of artificial cultivation. This conclusion may be drawn from the fact that tests carried out simultaneously with several strains, and repeated with the following generations, did not show a uniform reduction or augmentation of the agglutinating values, which would follow if an alteration of the serum had occurred. While this variability is a prominent feature, it must not be inferred that all of our meningococcus strains were equally erratic in this respect. Some of our strains have preserved the same degree of agglutinability for considerable periods. Aside from the inconvenience arising from this peculiarity of the meningococcus in the course of systematic experimentation with this organism, it is of very considerable practical importance. Obviously, meningococcus strains exhibiting this peculiarity to a marked degree cannot be used for serum diagnosis. To overcome this difficulty von Lingelsheim[9] suggested the use of killed cultures, a procedure which we can endorse in view of our own experience with animal sera, and in view of the discordant results which we obtained with human sera acting on the living microörganisms. These results will be considered in the latter part of this section.

Aside from variations of the agglutinability of individual strains, we have found great differences in the degree of agglutinability exhibited by different strains in the presence of the same immune serum. Similar observations have been recorded by Dunham,[30] Kolle and Wassermann,[46] Jochmann,[43, 44] Kutscher,[47] Ditthorn and Geldemeister,[48] Jacobitz,[49] Eberle,[45] and others. Table No. VII. records the results obtained with sixty-five strains exposed to the action of a serum derived from a rabbit immunized against meningococcus strain M. 30. None of these strains responded to the action of normal sera diluted above 1–50; the vast majority failed to react in normal serum dilutions of 1–10. A review of this table shows that twenty-five strains, or, approximately, forty per cent, either failed to react wholly or yielded incomplete reactions in dilutions of 1–50. On the other hand, three strains yielded positive results in dilutions of 1–3,000. The remaining strains occupied an intermediate position. The homologous strain in this instance was only moderately agglutinable, reacting in dilutions of 1–1,000. Agglutination tests with more powerful sera yielded similar results. In the course of our investigations we have, in two instances, succeeded in producing a serum in rabbits which agglutinated the homologous strain in dilutions of 1–25,000, yet sera of such unusual potency failed to influence certain unquestionable meningococcus strains in dilutions sufficiently high to exclude the action of normal agglutinins. Recently isolated strains as a rule were less sensitive to the action of agglutinins than were older cultures, suggesting that passage through the animal body exerts an unfavorable influence on this property of the meningococcus.

In the course of this paper we shall refer to strains which failed to react to powerful immune sera in dilutions below 1–100 as inagglutinable strains. Many of the strains included in this category failed to react to immune sera in dilutions of 1–10, while others reacted in dilutions of 1–50 and were, therefore, strictly speaking, not inagglutinable. However, in very concentrated sera we are not certain that the effect produced is due to the interaction of specific substances and, furthermore, serum derived from some normal rabbits

" 89	Throat	±	±			
" 71	Spinal fluid	±				
" 63	" "	±	±	±	±	±
" 70	" "	±				
" 61	" "	±				
" 175	Nose	±	±			
" 73	Spinal fluid	±	±	±	±	
" 74	Blood	±	±	±		
" 176	Nose	±	±			
" 177	Throat	±	±			
" 7	Spinal fluid	O				
" 178	Throat	O				
" 79	Spinal fluid	O				

± = Partial reaction.

us strain.

nal Rabbit Serum.

	Gonococcus Strain.						
500	Normal Salt Solution Control.	10	25	50	100	250	500
	O	O					
	O	O					
	O	O					
	O	O					
	O	—	—	—	—	—	
	O	O					
	O	O					
	O	O					
	O	O					
	O	—	—	—			
	O	O					
	O	O					
	O	O					
	O	O					
	O	—	—	—	—	—	

agglutinated meningococcus strains in dilutions of 1–50, and in rare instances even somewhat higher (1–250). The occurrence of partial reactions of equal intensity in successive dilutions, which we have attributed to inequalities in the agglutinability of different individuals in the same culture, is particularly common with the so-called inagglutinable strains. The reactions with strains M. 53 and M. 63 may be pointed out as showing this peculiarity to a marked degree.

The relatively large number of inagglutinable strains encountered by other observers and ourselves renders this method unfit for purposes of identification and differentiation. While this is true in the main, the tests recorded in Table No. VIII. (opposite) show that even though the agglutinability of a meningococcus strain is much impaired, it may still be possible to differentiate such a strain from other cocci responding to a meningococcus immune serum to a greater or less extent by observing the rate of the reaction.

Rate of the reaction.—If we compare the results designated as Series I. we find that the gonococcus is agglutinated by a meningococcus immune serum in higher dilutions than a genuine meningococcus strain, but when we consider the rate of the clumping it becomes evident that the two reactions are not identical. These differences in the course of the reaction may be due either to differences in the substances interacting, or to peculiarities of the organisms concerned. The latter proposition is not borne out by the facts. The tardy appearance of the clumping in the case of the gonococcus cannot be attributed to a lack of agglutinability on the part of the organism, since the end reactions show that it is more sensitive to the influence of the meningococcus immune serum than is the homologous strain, nor to any peculiarity of the organism in the presence of sera generally for it responds promptly, as shown by other tests, to the action of the homologous immune serum. We are led, therefore, to ascribe the differences to the substances interacting. As to the nature of these differences, the reactions with normal sera furnish a clue. If we compare the reaction of the gonococcus to meningococcus immune serum with that occurring in normal serum, we find that they are identical (Series III.) or nearly so (Series II.). We may infer, therefore, that the substances participating in these reactions are identical. It follows from this that the gonococcus was not specifically influenced by the meningococcus immune serum.

The specificity of the reaction between the meningococcus and the corresponding serum is suggested though not proved by the failure of this organism to respond to the action of normal serum. Proof as to the specificity of the reactions with the meningococcus strains was furnished by absorption tests. Before leaving this table we may call attention to the fact that if the four-hour readings are taken as a standard for comparison, a differentiation of these strains would be possible.

While the prompt response of relatively inagglutinable meningococcus strains to the action of the corresponding immune serum usually holds true, we are able to cite exceptions to this rule. Moreover, occasionally meningococcus strains which are not particularly agglutinable react promptly to the influences of other immune sera, although to a less extent than to their own immune serum. Table No. IX. (opposite) serves to record such exceptions to the general rule.

Series I. shows the prompt agglutination of a meningococcus strain in the presence of a gonococcus immune serum, and Series II. shows that a meningococcus strain may be equally slow in responding to the action of meningococcus immune serum, normal serum, and gonococcus immune serum. The course of the reaction in the latter instance suggests that even in the corresponding immune serum the meningococcus was not specifically influenced. Concerning the first series we may say that we are in possession of experimental evidence which would explain the occurrences noted, but a discussion of these experiments would lead us beyond the scope set for this paper.

Th

Menin-gococcus Strains.		Normal Salt Solution Control.	Me it's Serum. 25	50	100	250	500
Series I.	2 hours.	O	▬				
	3 "	O	▬				
	4 "	O	▬				
	24 "	O	▬	▬	±		
Series II.	2 hours.	O	O				
	3 "	O	±				
	4 "	O	▬				
	24 "	O	▬				

strains, a pseudomeningococcus and a gonococcus strain.

Normal Rabbit's Serum.

		55° C.						
500	1000	10	25	50	100	250	500	1000
		0						
		±						
		—						
		—	—	—				
		0						
		0						
		0						
		±	±					
		0						
		0						
		±						
		±	±					
		0						
		0						
		0						
		—						
		0						
		0						
		0						
		±						
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		0						
		0						
		0						
		0						
		0						
		0						
		0						
		0						
		0						
		0						
		0						
		—	—	—				

Since we shall not refer to this phase of the subject again, we may add that similar differences between the rate of the reactions induced by normal and specific agglutinins were noted in connection with tests made with normal and with immune sera produced by injections of the allied Gram negative cocci.

Influence of temperature on the reaction. — At this juncture we may stop to consider the method designed by Kutscher[47] to overcome the diagnostic difficulties arising from the inagglutinability of certain meningococcus strains. This observer found that a meningococcus strain, which was relatively inagglutinable when tested at 37° C., reacted to immune sera in rather high dilutions when the tests were carried out at 55° C. Our own experiments carried out in the manner indicated by this observer showed that under these conditions the velocity of the reaction was enhanced, but that this was not associated with any pronounced or invariable augmentation of the extent of the reaction. Table No. X. (opposite) serves to record the result thus obtained.

We have repeated these experiments with other strains acted upon by other immune sera, and have found slight variations in the results, but at no time have we observed any marked augmentation of the values obtained at 55° over those obtained at 37° C. We are, therefore, unable to corroborate the findings of Kutscher.

Eberle[45] reported a series of parallel investigations with results which are in accord with our own. Among eighteen different meningococcus strains he found that some strains yielded better results when the tests were carried out at 56° C., whereas others reacted in higher serum dilutions at 37° C. Moreover, none of the strains which yielded doubtful results at one temperature gave positive reactions when exposed to another temperature.

A comparative study of the influence of incubator, room, and ice-box temperature on agglutination reactions of meningococci exposed to immune serum yielded the results recorded in Table No. XI. (opposite).

A review of this table shows that the end results are not markedly influenced by temperature. While a favorable influence of incubator temperature is apparent in three instances, two other strains responded to a greater extent at room temperature. Whether the results obtained with these latter strains are due to the interaction of specific substances is open to question. Other tests designed to determine the influence of different temperatures on the rate of the clumping clearly showed that incubator temperature as a rule hastens the reactions.

Spontaneous impairment of the agglutinability of the meningococcus. — Concerning the spontaneous impairment of the agglutinable properties of the meningococcus, our investigations yielded very little definite information.

Media containing serum exerted a deleterious influence on the agglutinable properties of these organisms. For this reason our strains were transferred to and maintained on serum free media the moment they could be induced to grow well on such media. The following experiments serve to indicate the profound influence which the culture medium may exert on the agglutinable properties of this organism.

Two meningococcus strains (M. 250 and M. 2451) were grown in glucose bouillon for two generations for the purpose of augmenting their agglutinable properties. These strains were then transferred to glucose agar, plain agar, and ascitic agar. Agglutination tests undertaken with the first generation on solid media yielded the following results:

The comparative e

Strain.
M. 83
M. 9
M. 51
M. 101
M. 19
M. 61
M. S AA
M. 66
M. 71

Comparison of values obtained after the lapse of two hours:

	Strain M. 250.	Strain M. 2451.
Glucose agar culture	++ 1-2000>	+ 1-100
Plain agar culture	+ 1-100	+ 1-50 .
Ascitic agar culture.....................	+ 1-50	+ 1-25

Comparison of values obtained after the lapse of twenty-four hours:

	Strain M. 250.	Strain M. 2451.
Glucose agar culture	++ 1-2000>	+ 1-250
Plain agar culture	+ 1-500	+ 1-250
Ascitic agar culture.....................	+ 1-500	+ 1-250

According to these tests glucose agar preserved the agglutinable properties of the two strains employed, whereas plain agar and ascitic agar exerted an unfavorable influence. In the case of strain M. 250, both the rate of the reaction and its extent have been materially altered by the medium employed for the cultivation of this strain. The effects of the culture medium are less obvious in the case of strain M. 2451. It is possible that these differences are due to the fact that the susceptibility of strain M. 250 to the action of agglutinins was recently acquired, *i.e.*, brought about by its passage through bouillon, whereas in the case of strain M. 2451 the degree of agglutinability was not materially altered by this treatment. It seems rational to assume that recently acquired properties are more easily disturbed by external influences than those possessed by an organism for considerable periods.

Experiments designed to determine which component of the serum exerted the prejudicial influence referred to yielded no satisfactory solution of the problem. Similar observations were made by Collins[50] on the influence of serum media on the agglutinable properties of the pneumococcus.

Augmentation of the agglutinability of the meningococcus. — Passage through bouillon, as we have already indicated, increased the agglutinability of some of our strains. The broth employed for this purpose was prepared according to standard methods. The reaction which appeared to be most favorable in bringing about the desired results ranged between +.5 per cent and + one per cent to phenolphthalein. Both plain and glucose broth were employed. Concerning the relative values of these two varieties of broth, experiments showed that glucose broth is to be preferred for this purpose. While several strains reacted promptly to this treatment, the agglutinability of others was not materially altered, even after prolonged growth in fluid media. In a few instances a reduction of the agglutinability followed.

As indicating the prompt augmentation of the agglutinability of a relatively inagglutinable strain which may be brought about by passage through bouillon, we may cite the following example:

Strain M. 250, which failed to react to the influence of an homologous serum in dilutions above 1–250, responded to the action of the same serum in dilutions of 1–4,000 after remaining in bouillon for one generation (twenty-four hours). The subsequent generation reacted in dilutions of 1–8,000. In order to exclude any influence which the bouillon might exert on the reaction, these tests were made with the growth taken from glucose agar transplants (first generation). We may add that the glucose agar employed served also for the growth of the controls, which showed no material alteration of their clumping properties.

The absorption tests recorded in the following table (XII.) were undertaken to determine the absorptive capacities of a meningococcus strain which had been sensitized by passage through bouillon and those of the same strain maintained on solid media. Strain M. 250, passed through bouillon for two generations and transferred to solid media, responded to the action of an immune serum in dilutions of 1–3,000, whereas the same strain grown on solid media reacted in serum dilutions of 1–1,000 only. It is of interest to note that passage through bouillon did not result in a sensitization for normal agglutinins.

Table No. XII.

Absorption of a meningococcus immune serum by a "sensitized" and a control meningococcus strain.

Serum.	Strain Employed for Absorption.	Agglutination with Serum M. 250 "16."	Dilution of Serum Employed for Absorption.	Time.	Temperature.	Strain Employed for Testing Absorbed Serum.	Agglutination with Unabsorbed Serum.	Agglutination with Absorbed Serum.	Percentage of Agglutinins Absorbed.
M. 250 "16."	M. 250 sensitized.	1–3,000	1–50	2 hrs.	37° C.	M. 250 sensitized.	1–3,000	1–2,000	33.33%
	M. 250 control.	1–1,000	"	" "	"	M. 250 sensitized.	"	"	"

According to the records, the increased agglutinability was not associated with increased binding capacities. In this instance, therefore, only one portion of the agglutinable molecule was favorably influenced by the liquid medium. Further experiments along these lines are required to show whether this is invariably the case.

Concerning the nature of the changes produced by broth we are not inclined to attribute them to any specific influence of the bouillon as such, nor to the fact that it is a liquid medium. The sensitization is not a temporary one, but is perpetuated for many generations if the organism is transferred to suitable solid media. It is, moreover, well known that transplantation of an organism, maintained for long periods on solid media, to a liquid menstruum, tends to rejuvenate the strain. Very often the original characteristics which have faded away during artificial cultivation on solid media may be recovered by this expedient.

AGGLUTINOGENIC PROPERTIES OF THE MENINGOCOCCUS. — While the organisms referred to by us as inagglutinable meningococci are morphologically, culturally, and biochemically indistinguishable from those which react promptly to immune sera, the identity of these two varieties remains to be proven. The absorption tests, which will bear a part of the burden of proof, will be considered later.

In the present section we propose to record the experiments designed to determine the agglutinogenic properties of the agglutinable strains as compared with those enjoyed by relatively inagglutinable strains. For this purpose we selected two agglutinable and three inagglutinable strains.

Two of these latter (M. 101 and M. 175) were inagglutinable when first selected for the present purpose, but subsequently reacted to certain immune sera in dilutions of 1–100. The strain designated as M. 61, while it reacted to normal sera in dilutions of 1–25, was refractory to the action of specific sera in dilutions above 1–50.

The animal experiments designed for the purpose indicated were carried out in the following manner: Five rabbits, approximately equal in size and weight, received weekly intravenous injections of the corresponding strains, beginning with .002 gram and terminating with .006 gram of moist bacteria killed at 65° C. for thirty minutes. Eight days after the fourth injection the animals were bled and the agglutinating value of each serum determined.

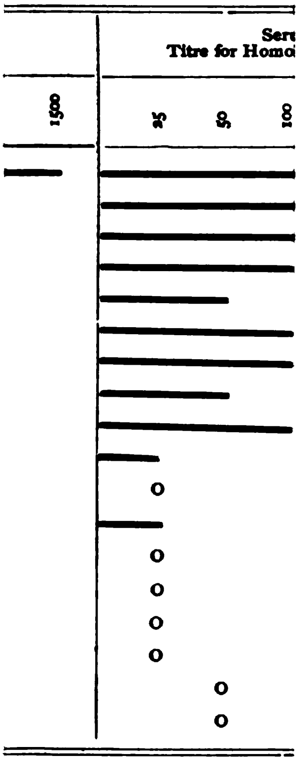
The results yielded by these experiments are recorded in Table No. XIII. (opposite).

A review of this table furnishes the following data:

1. All the strains employed for this experiment produced, when introduced into rabbits, immune sera which affected agglutinable meningococcus strains in fairly high dilutions. We may conclude, therefore, that the strains selected for our experiments belong to the same species, although the agglutination reactions alone failed to establish their identity.

2. In comparing the agglutinating values of the sera under consideration, it will be found that although the most agglutinable strains produced the most effective sera, a

normal rabbit's serum versus m



definite relationship between agglutinability and agglutinogenic capacity does not exist. Strain M. 101 is practically inagglutinable, yet it produced a serum only a trifle less effective than that produced by the very much more agglutinable strain M. 83. On the other hand, strain M. 175, which reacted to an immune serum to the same extent as did strain M. 101, produced a less effective serum. Finally, strain M. 61, which failed to react to immune sera in sufficiently high dilutions to exclude the action of normal agglutinins, yielded a fairly effective serum; approximately equal to that produced by strain M. 175. That these differences are not solely due to individual peculiarities of the animals employed for these experiments is borne out by the absorption tests which will be considered presently.

3. The failure to react to an immune serum on the part of a strain is not due to any peculiarity of the serum employed for the test. Since sera produced by the injection of relatively inagglutinable strains are found to be less effective in their action on such strains than are more powerful sera produced by the injection of agglutinable strains, we must infer that the fault lies in great part, at least, with the strain.

Finally, we wish to call attention to certain qualitative differences found in the several sera examined. If we set aside for the moment slight differences which may be considered within the limits of experimental error, we find that sera M. 83 and M. 24, although equally effective when judged by their action on agglutinable strains, act differently on some of the inagglutinable strains. Serum M. 83 embraces in its action all the strains tested with but one exception, whereas serum M. 24 yields negative results with seven strains in dilutions of 1–25. Serum M. 101, though apparently weaker than M. 24, also acts on most of the strains tested.

While a number of hypotheses might be offered to explain these differences, we have no definite experimental evidence which might serve this purpose. Experiments having for their object a solution of this problem are still under way.

Comparison of the absorptive capacities and agglutinogenic properties of different meningococcus strains. — The following table (No. XIV.) records the binding capacities of the strains employed for the preceding experiments:

TABLE NO. XIV.

Relative absorptive capacities of meningococcus strains employed for the production of the immune sera, M. 24, M. 83, M. 101, M. 175, and M. 61.

Number of Strains Employed for Absorption.	Sera Employed for Absorption.	Agglutinability of Strain Employed for Absorption.	Dilution of Sera Employed for Absorption.	Strains Employed for Testing Unabsorbed and Absorbed Sera.	Titre of Unabsorbed Serum.	Titre of Serum after Absorption.	Percentage of Agglutinins lost by Absorption.
M. 24	Serum M. 24	1-4,000	1-50	M. 24	1-4,000	1-100	97.5%
" 83	" " 83	1-1,800	1-50	" 83	1-1,800	O 1-100	100%
" 101	" " 83	1-100	1-50	" 83	1-1,800	± 1-100	97.2%?
" 175	" " 175	1-100	1-25	" 83	1-250	1-50	80%
" 61	" " 61	O 1-50	1-25	" 83	1-250	1-250	0%

In comparing this table with the preceding one it will be noticed that the binding capacities exhibited by the several strains employed for immunization correspond in the main with their capacity to produce agglutinins in the animal body. The three strains, M. 24, M. 83, and M. 101, exhibiting the greatest absorptive capacity, yielded, when introduced into animals, the most powerful sera. If we make some allowances for individual differences in the animals employed for these experiments, we may say that strains M. 83 and M. 101 were endowed with, approximately, the same agglutinogenic capacities. These strains also exhibited, approximately, the same absorptive capacities, although they differed markedly as regards agglutinability. Strain M. 61 yielded wholly unexpected results. Judged solely by absorption tests, this strain having lost the power to combine with specific agglutinins should, according to prevailing views, recently questioned, however, by Landsteiner, be wholly devoid of agglutinogenic powers. This, however, was not the case. A glance at Table No. XIII. shows that injections of this organism resulted in the production of a serum capable of agglutinating one strain in dilutions of 1–1,000. It must be mentioned that the suspensions of this strain employed for immunization were not tested for their absorptive capacities, which robs this experiment of much of its value. In view of the fluctuations in absorptive capacities exhibited by these organisms, it is quite possible that one of the cultures used for immunization of the animal had regained its combining capacities, and that the grade of immunization produced is referable to this particular culture. One other possibility may be mentioned. The number of agglutinins absorbed by this strain may have been too small to be appreciable by the methods employed by us. Unfortunately, an accident to our incubator resulted in the death of this strain before this experiment could be repeated, with proper controls, to eliminate the sources of error referred to. While, therefore, we must admit the possibilities suggested above, further experiments along these lines with artificially altered strains furnished conclusive proof that failure to combine with specific agglutinins in a test-tube does not signify total loss of agglutinogenic properties. It follows, therefore, that negative absorptive tests cannot be accepted with the same lack of reserve as has been the custom in the past. A discussion of these experiments to prove the validity of this assertion would, however, carry us beyond the limits set for this paper.

Agglutination tests. Normal rabbits' sera versus meningococcus strains. — The necessity for normal serum controls in carrying out agglutination tests with this group of organisms has furnished us with records concerning the action of normal sera taken from, approximately, one hundred animals.

The results obtained may be summarized as follows: While certain very agglutinable strains responded to the action of normal rabbits' sera in dilutions as high as 1–250, this is on the whole a rare occurrence. As a general rule, positive reactions were not obtained in serum dilutions above

1–25, and very many strains failed to react in dilutions of 1–10. In a series of tests carried out simultaneously with sera from four normal rabbits, the following results were obtained: Of forty-six strains tested, eight failed to respond to the action of these sera in dilutions of 1–10; eighteen yielded incomplete reactions in the same dilutions; thirteen yielded positive reactions in dilutions of 1–10 and partial reactions in dilutions of 1–25. Finally, seven strains gave positive reactions in dilutions of 1–25 and partial reactions in dilutions of 1–50. The agglutinating values obtained with the four normal sera acting on the same strain were practically identical, *i.e.*, each strain was influenced to the same extent by all four sera. This, however, is not the rule. On the contrary, the normal agglutinin content of sera taken from different animals varies considerably. In comparing the figures given above, very considerable differences in the agglutinability of different strains will be observed. In general it may be stated that strains which were very sensitive to the action of specific agglutinins were also rather agglutinable in the presence of normal sera.

Tests made with the same strains at varying intervals also showed marked variations in their behavior toward normal agglutinins.

Agglutination tests. Heterologous immune sera versus meningococcus strains, meningococcus immune sera versus heterologous strains, and the reactions of the allied Gram negative cocci in the presence of the corresponding immune sera. — In this section we propose to consider the action of heterologous and homologous immune sera on the various groups of Gram negative cocci. It will be convenient to include remarks regarding the agglutinogenic properties exhibited by these organisms and the effects of normal sera on these strains. To facilitate comparisons we have usually recorded the results obtained with several sera in the same table. These are not composite tables in the sense that records of experiments undertaken at different times are compiled. In each instance a given strain was tested simultaneously with the several sera included in one table. One exception to this rule will be found in the table recording reactions with the micrococcus catarrhalis. Here we have recorded the values obtained with normal serum derived from the animal employed for the production of the immune serum.

Agglutination tests. Pseudomeningococcus and meningococcus immune sera versus pseudomeningococcus and meningococcus strains. — While the pseudomeningococcus usually formed stable suspensions and offered no difficulties in so far as the technical procedures were concerned, we encountered great difficulties in producing immune sera with these organisms. All of them were decidedly more toxic for rabbits than were meningococcus strains. Many of our animals succumbed to very small doses of the killed organisms, and those that survived the injections yielded sera which on account of the low grade of immunity were unfit for purposes of differentiation. These experiences suggest that the agglutinogenic properties of these organisms are limited. This may, however, only apply when rabbits are employed for this purpose. After repeated trials with all the strains in our possession we finally succeeded in obtaining a serum which answered the requirements. This serum, however, influenced only the strain employed for its production; none of the other strains responded to its action. While this in itself does not predicate the non-identity of the members of this group, absorption tests, which will be considered later, suggest that at least two varieties of organisms have been included under the heading " pseudomeningococci."

While, therefore, uncertainties exist concerning the relationship of the various members of this group to each other, the results of the following experiments taken in conjunction with the results of absorption tests justify their separation from genuine meningococci.

The following table (No. XV.) records the results obtained with a pseudomeningococcus immune serum, a meningococcus immune serum, and a normal serum acting on pseudomeningococcus and meningococcus strains:

TABLE NO. XV.

Agglutination reactions. Pseudomeningococcus and meningococcus immune sera and a normal rabbit's serum versus pseudomeningococcus and meningococcus strains.

		Pseudomeningococcus Serum. P.M. 2. Titre for Homologous Strain 1–500.								Meningococcus Serum. M. 250. Titre for Homologous Strain 1–5,000.								Normal Rabbit's Serum.				
		25	50	100	250	500	1000	2500	5000	25	50	100	250	500	1000	2500	5000	25	50	100	250	500
Pseudomeningococcus Strains.	P.M. 2 . . .	▬	▬	▬	▬	▬				▬	▬							▬	▬			
	" 1 . . .	±								±								±				
	" 3 . . .	±								±								±				
	" 4 . . .	O								O								O				
	" 5 . . .	O								O								O				
Meningococcus Strains.	M. 250	▬	▬	▬						▬	▬	▬	▬	▬	▬	▬	▬	▬	▬	▬		
	" 252	O								▬	▬	▬						O				
	" 101	O								▬	▬							O				
	" 253	O								▬								O				
	" 251	O								▬								O				

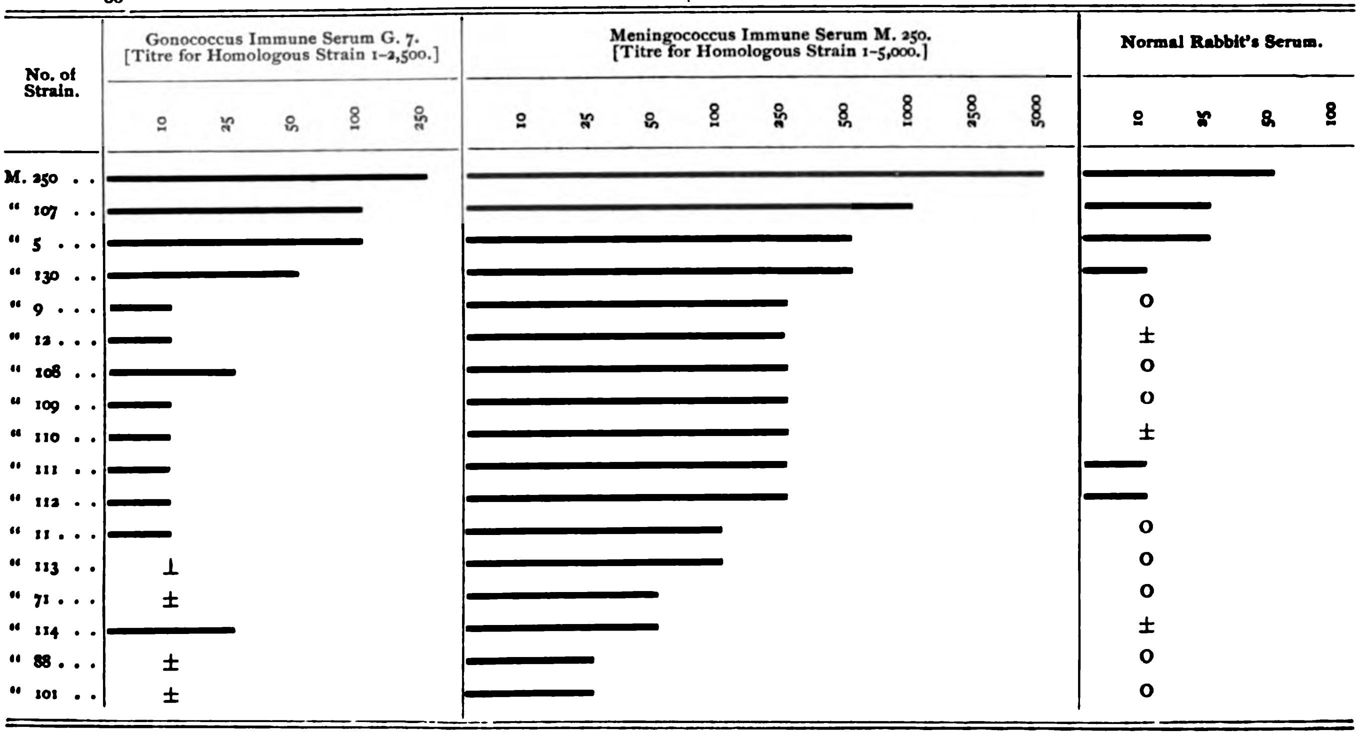
± = Partial reaction.

In reviewing these results it will be observed that the action of the meningococcus immune serum on pseudomeningococcus strains is identical with that of normal rabbits' serum. We may, therefore, infer that the pseudomeningococcus strains were not specifically influenced by the meningococcus immune serum. The table further shows that the converse also applies. Only one meningococcus strain responded to the action of pseudomeningococcus immune serum and the extent of this reaction does not exceed that produced by normal serum. Whether a group relationship is demonstrable by means of serum reactions will be considered in connection with absorption experiments. In reviewing the reactions of the pseudomeningococcus serum with the various members of the corresponding group, we find that the homologous strain alone responds to its action. Finally, the table emphasizes the incompetency of agglutination reactions for purposes of differentiation. For example, strain M. 101, the meningococcic nature of which has been established, responds to the action of a meningococcus immune serum to the same extent as does the pseudomeningococcus strain P.M. 2. Moreover, the meningococcus strains M. 253 and M. 251 are less sensitive to the action of a meningococcus immune serum than is the pseudomeningococcus strain P.M. 2.

Agglutination test. Meningococcus and gonococcus immune sera versus meningococcus and gonococcus strains.— Table XVI. serves to record the results obtained with a gonococcus immune serum, a meningococcus immune serum, and a normal rabbit's serum acting on a series of meningococcus strains exhibiting different degrees of agglutinability.

TABLE NO. XVI.

Agglutination reactions. Gonococcus and meningococcus immune sera and a normal rabbit's serum versus meningococcus strains.



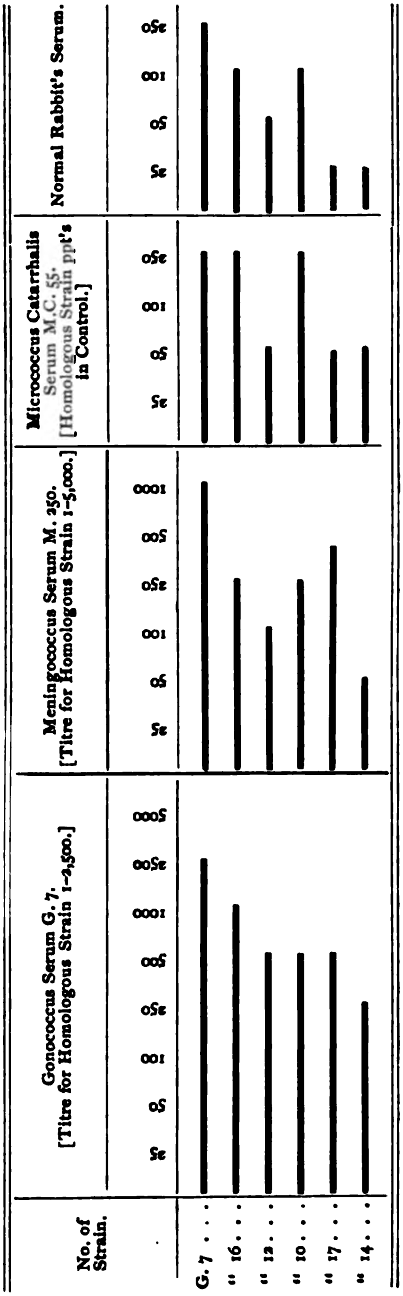
± = Partial reaction.

A comparison of the results contained in this table shows that in every instance the meningococcus strains reacted to the meningococcus serum in higher dilutions than to the gonococcus and normal sera. If we compare the results obtained with gonococcus immue serum acting on meningococcus strains and those obtained with normal rabbits' serum, the presence of group agglutinins in the former is clearly indicated. It will be noticed, however, that the action of this group factor is only discernible in connection with the more agglutinable meningococcus strains. It would appear, therefore, from the results here recorded that a reduction of the agglutinability of a strain for specific agglutinins is attended as a rule by a similar reduction for group agglutinins.

Table XVII. serves to indicate the results obtained with a meningococcus immune serum, a gonococcus immune serum, micrococcus catarrhalis immune serum, and a normal serum acting on a series of gonococcus strains.

TABLE No. XVII.

Agglutination reactions. Gonococcus, meningococcus and micrococcus catarrhalis immune sera and a normal rabbit's serum versus gonococcus strains.



We have selected this table from a number of others covering the same field, because it shows clearly some of the difficulties which may be encountered in differentiating a gonococcus from a meningococcus and, also, because it shows the close relationship of these species to each other, a fact which has been noted by many previous observers.

The values obtained with the micrococcus catarrhalis serum are slightly higher than those obtained with normal serum. We are unable to say whether this difference in the sera derived from the two animals in question was brought about by the injection of micrococcus catarrhalis into one of them or is solely due to individual peculiarities of the animals employed.

The results recorded in this table emphasize the necessity of employing absorption tests to differentiate these organisms. Such tests clearly show that these organisms are specifically influenced only by their respective sera. It may be added that similar agglutination tests with the same strains and with other gonococcus and meningococcus immune sera yielded results which differed sufficiently to serve for differential purposes.

Before leaving this subject we may remind the reader of the work of Torrey[51] concerning serum reactions with the gonococcus. This observer found that the organisms which pass as gonococci are not identical when judged by their agglutinogenic and absorptive capacities. Since this work was carried out simultaneously with our own and with the same strains employed by us, we felt that parallel investigations on our part could be omitted. We are, therefore, not in possession of conclusive experimental evidence which would confirm or disprove Torrey's contentions.

We further wish to state that in the course of our investigations we found that the gonococcus is extremely sensitive to the action of both normal and group agglutinins.

Agglutination tests. Micrococcus catarrhalis immune serum versus meningococcus strains. — The micrococcus catarrhalis immune serum employed for these tests agglutinated the homologous strains in dilutions of 1–1,000. The meningococcus strains selected were for the most part relatively inagglutinable in the presence of meningococcus immune serum. Table No. XVIII. (opposite) records the results obtained.

Judging from the results obtained in this series of tests, the meningococcus strains are not influenced by micrococcus catarrhalis immune sera in dilutions sufficiently high to exclude the action of normal agglutinins. While in this series a few strains reacted positively in dilutions of 1–50 and partially in dilutions of 1–100, the majority of the strains gave negative results in dilutions of 1–50. In a series of tests not included in the above table we have, in a few instances, obtained somewhat higher values with micrococcus catarrhalis immune sera. While no relationship between the micrococcus catarrhalis and the meningococcus is apparent in these reactions, it must be borne in mind that the sera employed by us for this purpose were not powerful.

Agglutination tests. Meningococcus immune serum versus micrococcus catarrhalis strains. — Agglutination tests carried out with the micrococcus catarrhalis are attended by very considerable, if not insurmountable, difficulties. In a collection of micrococcus catarrhalis strains, comprising some sixty different specimens, we encountered only two which formed stable suspensions in normal salt solution. All the others, though tested repeatedly, sedimented spontaneously in the control normal salt solution and in the various serum dilutions. Moreover, the two exceptions to the rule referred to above did not retain their acquired stability in suspensions for any considerable period. Our information, therefore, concerning the reaction of these organisms to normal and immune sera is somewhat meager. Five meningococcus immune sera, varying in their agglutinating values (agglutinating index for meningococcus strains 1–4,000, 1–4,000, 1–1,500, 1–1,000, and 1–500 respectively) were tested on the two strains referred to. The values obtained were no

		Immune Produced by [1:500.]			
		10	25	1000	1500
Chromogenic group 1.	L.Y. 1 I.	━━━	━━━		
	" 2 I.	━━━	━━━		
	" 6 I.	━━━	━━━		
	" 3 I.	━━━	━━━		
	" 7 I.	━━━	━━━		
	" 4 I.	Sedimented sp			
	" 5 I.	"			
Chromogenic group 2.	L.Y. 8 II.	±	±		
	" 11 II.				
	" 9 II.	Sedimented sp			
	" 10 II.	"			
Chromogenic group 3.	L.Y. 12 III.	±	±	━━━	━━━
	" 13 III.	━━━	±		
	" 15 III.	━━━	±		
	" 16 III.	━━━	━━━		
	" 14 III.	±	±		
	" 17 III.	━━━	━━━		
	" 18 III.	Sedimented sp			
	" 21 III.	"			
	" 22 III.	"			

higher than those obtained with normal sera, viz., positive reactions in dilutions of 1–25 or lower. Concerning the agglutinogenic capacities of the micrococcus catarrhalis we may add that several strains injected into rabbits yielded sera of moderate strength (agglutinating indices 1–500–1–1,000).

Agglutination tests. Members of the chromogenic groups I., II., III. — Many of the strains included under these headings showed a more or less pronounced tendency to spontaneous sedimentation in normal salt solutions and in various dilutions of normal and immune serum. The fact that this lack of stability in suspensions is not a constant factor, but varies from generation to generation, not only adds greatly to the difficulties surrounding agglutination experiments with these strains, but renders extreme caution necessary in the interpretation of the results obtained.

Agglutination tests. Immune sera, chromogenic groups I., II., III. versus chromogenic groups I., II., III. — A review of Table No. XIX. (opposite) shows that it is possible to distinguish the chromogenic groups from each other by means of agglutination tests, providing the tests are made simultaneously with sera corresponding to each group. It will be observed, however, that very considerable differences in the agglutinabilities of the strains exist, and that identification of the relatively inagglutinable is not possible in the absence of suitable control tests. We may also mention the fact that since these tests were undertaken we have isolated a considerable number of strains which according to their fermentative capacities and other peculiarities correspond to the three groups under discussion, yet absorption tests fail to establish their identity.

Agglutination tests. Meningococcus, gonococcus, and micrococcus catarrhalis immune sera versus chromogenic groups I., II., III. — The results recorded in the following tables (XX. and XXI.) indicate that none of the chromogenic

Gram negative cocci bear any relationship to the meningococcus, gonococcus or micrococcus catarrhalis in so far as agglutination tests are competent to establish this point. The values obtained in this instance with normal serum acting on members of the chromogenic group are unusually low. Very frequently these strains responded to the action of normal sera in dilutions of 1–25 or 1–50.

TABLE No. XX.

Agglutination reactions. Meningococcus, gonococcus and micrococcus catarrhalis immune sera and a normal rabbit's serum versus strains from chromogenic groups I., II., and III.

		Meningococcus Immune Serum M. 250 "74." Titre for Homologous Strain 1-5,000.			Gonococcus Immune Serum "G. 7." Titre for Homologous Strain 1-2,500.			Micrococcus Catarrhalis Immune Serum "T. 1." Homologous Strain pp'ts in Control.			Normal Rabbit's Serum.		
		10	25	50	10	25	50	10	25	50	10	25	50
Chromogenic group 1.	L.Y. 1 I.	±			±			±			±		
	" 2 I.	±			O			▬	±		±		
	" 3 I.	±			O			±			±		
	" 4 I.	±			▬			O			±		
	" 5 I.	±			±			O			▬	±	
	" 6 I.	O			O			±			O		
	" 7 I.	±			O			±			O		
Chromogenic group 2.	L.Y. 11 II.	±			±			▬	±		±		
Chromogenic group 3.	L.Y. 12 III.	±			±			▬	±		▬	±	
	" 13 III.	±			±			±			±		
	" 14 III.	±			±			±			±		
	" 15 III.	±			±			▬	±		▬	±	
	" 16 III.	±			▬	±		±			±		
	" 17 III.	±			±			O			±		

± = Partial reaction.

TABLE No. XXI.

Agglutination reactions. Immune serum produced by members of chromogenic groups I., II., and III. versus meningococcus, micrococcus catarrhalis, and gonococcus strains.

	L.Y. 1. Immune Serum Chromogenic Group I. Titre for Homologous Strain 1-1,000.						L.Y. 8. Immune Serum Chromogenic Group II. Homologous Strain Agglutinates Spontaneously.						L.Y. 12. Immune Serum Chromogenic Group III. Titre for Homologous Strain 1-1,500.						Normal Rabbit's Serum.					
	10	25	50	100	250	500	10	25	50	100	250	500	10	25	50	100	250	500	10	25	50	100	250	500
M. 250 .	▬	▬	▬	▬			▬	▬	▬				▬	▬	▬				▬	▬	▬			
" 11 .		±	±				O						O						±					
M.C. 60	O						▬	▬	±				±						±					
G. 7 . .	▬	▬	▬	▬			▬	▬	▬				▬	▬	▬	▬	▬		▬	▬	▬			
" 10 . .	▬	▬	▬				▬	▬	▬				▬	▬	▬				▬	▬				

± = Partial reaction.

Agglutination tests with the micrococcus pharyngis siccus and with the diplococcus mucosus were not undertaken. The former at no time formed a stable suspension in normal salt solutions, and the latter organism is so easily identified by other means that agglutination experiments with this organism seemed unnecessary for our present purposes.

Agglutination tests. Meningococcus immune serum versus diplococcus crassus and Jaeger "meningococcus." — The following table (XXII.) is inserted to show that a meningococcus immune serum has no specific agglutinating influence on the diplococcus crassus. The so-called Jaeger type of the meningococcus sedimented spontaneously in the immune and normal serum employed.

TABLE No. XXII.

Agglutination reactions. Meningococcus immune serum and a normal rabbit's serum versus the diplococcus crassus and the Jaeger meningococcus.

	Meningococcus Immune Serum M. 250. Titre for Homologous Strain 1-2,500.				Normal Rabbit's Serum.		
	10	25	50	100	10	25	50
Diplococcus crassus	▬				▬	▬	
Jaeger meningococcus	Spontaneous agglutination.				Spontaneous agglutination.		

ABSORPTION TESTS.

The object of these tests was three-fold: (1) To establish the identity of cultures which we have thus far regarded as belonging to the same species;

(2) To establish the relationship of the various groups to each other;

(3) To determine the relative absorptive capacities of different strains belonging to the same species, but exhibiting different degrees of agglutinability in the presence of homologous immune sera.

To meet these requirements the usual method of carrying out absorption (exhaustion) tests was modified accordingly. Instead of exhausting a serum with large and variable quantities of bacteria, each serum was absorbed with small and constant amounts in the hope of detecting slight differences in the absorptive capacities of different strains belonging to the same species. The suspension of bacteria employed was of the same density as that employed for the agglutination tests, viz., .004 gram of moist bacteria per cubic centimeter. This suspension was mixed with equal parts of diluted serum. The bacteria employed for extraction were therefore present in the proportion of .002 gram of moist bacteria per cubic centimeter. Of the two methods usually employed for the subsequent clarification of the absorbed serum, viz., filtration and centrifugalization, we selected the latter as being free from the objectionable features attending the use of the former method. Sources of error due to centrifugalization of an immune serum will be considered later.

Method. — An immune serum was diluted with normal salt solution to the desired extent and thoroughly mixed with an equal part of the standard suspension. Two other tubes which were to serve as controls received the same amount of the diluted serum plus equal parts of a normal salt solution. The series thus prepared was exposed to incubator temperature for two hours. Preliminary tests showed that the maximum absorption was attained within this time; twenty-four hours yielded no higher values. The tube containing the extracted serum and one of the serum control tubes were then centrifugalized for fifteen minutes (three thousand revolutions per minute). To distinguish the centrifugalized control tube from the second control sample we will refer to the former as the centrifuge-control, and to the latter as the incubator-control. This latter tube was exposed to room temperature during the process of centrifugalization.

The supernatant fluid of the centrifugalized specimens was now pipetted off and agglutination tests carried out in the usual manner with the extracted serum and the two control samples.

Influence of centrifugalization on the rate of the agglutination reactions. — Concerning the appearances of the extracted sera after centrifugalization, the following may be noted: In many instances centrifugalization resulted in a complete clarification of the liquid, in other instances the supernatant fluid was faintly turbid, though never sufficiently so to interfere with subsequent tests.

In comparing the mixtures extracted with agglutinable homologous strains and those absorbed by heterologous strains or by inagglutinable homologous strains, very distinct differences were noted. In the former instance, the organisms were firmly packed at the bottom of the tubes, the supernatant fluid was perfectly clear, and vigorous shaking of these tubes failed to reëstablish a perfect suspension. A tube thus treated had all the appearances of one showing a true agglutination reaction before complete sedimentation had occurred. In the case of the heterologous strains and of the inagglutinable homologous strains, the material was less firmly packed, the supernatant fluid was not quite clear, and vigorous shaking resulted in the formation of a perfect suspension.

In the former instance, therefore, we may infer that actual agglutination had occurred, whereas in the latter that sedimentation, due to centrifugal force, had alone taken place. These differences have recently been made use of by Brian[52] and also by Gaehtgens[53] to curtail the time ordinarily consumed in carrying out agglutination tests. According to the methods devised by these observers, suspected organisms are introduced into normal serum and immune serum dilutions graded in the usual manner. The series of tubes thus prepared are then centrifugalized for ten minutes. It was found that organisms which were susceptible to the influence of the immune serum were promptly agglutinated, and the end results corresponded with those obtained by the ordinary method of carrying out agglutination tests.

While we have not made a systematic study of this phase of the subject, our experience with inagglutinable strains lead us to believe that this method is applicable only to strains which respond readily to the action of agglutinins. This would limit the use of this method largely to serum diagnoses; for which a suitable strain can be provided.

Influence of centrifugalization on the agglutinins. — Before recording the results of our absorption experiments, the question as to the reliability of the method employed remains to be considered. Our reason for employing small and carefully measured quantities of bacteria for purposes of absorption have already been stated. One of the objections to the use of small quantities of bacteria is the fact that the number of agglutinins absorbed is restricted to rather narrow limits. We are, therefore, obliged to base our conclusions on the occurrence of only slight differences, a fact which renders absolute precision of our methods essential.

In the beginning of these investigations we carried out absorption experiments without a centrifuge-control, but soon discovered that the results obtained were unreliable. While, in many instances, we obtained what appeared to be reliable results, viz., that only such organisms absorbed specific agglutinins as belonged to the same species when judged by morphological, cultural, and biochemical properties, we not infrequently found that organisms which unquestionably belonged to a different species also caused a reduction in the number of specific agglutinins. For example, extraction of a meningococcus immune serum with a micrococcus catarrhalis strain was at times followed by a reduction in the number of specific agglutinins, whereas a second test carried out in the same manner revealed no diminution in the agglutinating value of the serum for meningococci. Moreover, absorption tests repeated with the same meningococcus strain acting on a meningococcus immune serum showed such marked variations that we were inclined to question the validity of our results. This lack of uniformity in the results obtained led to an investigation of the methods employed. Numerous tests with our standard suspensions satisfied us that the discrepancies observed were not due to any inaccuracies connected with the preparation of these suspensions. While variations in the strength of our suspensions no doubt existed, they were not sufficient to bring about any appreciable differences in the results of agglutination or absorption tests.

The possibility that improper cleansing of the tubes might be accountable for the reduction was considered, but special attention devoted to this part of the experiment failed to correct the error. We were led, therefore, to attribute the reduction to the process of centrifugalization. Introduction of centrifuge-control tubes revealed the fact that under certain conditions a considerable reduction of the agglutinating value of a serum occurs which is not due to the presence of microörganisms. The following tables (Nos. XXIII., XXIV., and XXV.) are designed to indicate the reduction of the agglutinating value of a serum in the process of centrifugalization and the erroneous results obtained in the absence of a centrifuge-control.

TABLE NO. XXIII.

Absorption of meningococcus immune serum.

Number of Strains Employed for Absorption.	Agglutination with Serum Employed.	Dilution of Serum for Absorption.	Time.	Temperature.	Strain for Testing Unabsorbed and Absorbed Sera.	Agglutination with Unabsorbed Serum.	Agglutination with Absorbed Serum.	Percentage of Agglutinins lost by Absorption.
M. 250	1–4,000 C.C.	1–250	2 hrs.	37°	M. 250	1–4,000 C.C.	1–500	87.5%
" "	1–6,000 I.C.	"	" "	"	" "	1–6,000 I.C.	"	91.66+%
M. 11	1–250	1–250	2 hrs.	37°	M. 250	1–4,000 C.C.	1–2,000	50%
" "	"	"	" "	"	" "	1–6,000 I.C.	"	66.66+%
G. 7	1–500	1–250	2 hrs.	37°	M. 250	1–4,000 C.C.	1–4,000	0%
" "	"	"	" "	"	" "	1–6,000 I.C.	"	33.33+%

In this table and in the following tables (XXIV. and XXV.) the upper line of each set gives the results obtained by using the centrifuge-control marked C.C. The lower line gives the results obtained by using the incubator-control marked I.C.

TABLE NO. XXIV.

Absorption of gonococcus immune serum.

Number of Strains Employed for Absorption.	Agglutination with Serum Employed.	Dilution of Serum Employed for Absorption.	Time.	Temperature.	Strain Employed for Testing Unabsorbed and Absorbed Serum.	Agglutination with Unabsorbed Serum.	Agglutination with Absorbed Serum.	Percentage of Agglutinins lost by Absorption.
G. 7	1–2,000 C.C.	1–250	2 hrs.	37° C.	G. 7	1–2,000	1–500	75%
" "	1–3,000 I.C.	"	" "	"	" "	1–3,000	"	83⅓%
G. 14	1–250	1–250	2 hrs.	37° C.	G. 7	1–2,000	1–1,000	50%
" "	"	"	" "	"	" "	1–3,000	"	66⅔%
M. 250	1–50	1–250	2 hrs.	37° C.	G. 7	1–2,000	1–2,000	0%
" "	"	"	" "	"	" "	1–3,000	"	33⅓%

TABLE NO. XXV.

Absorption of bacterium coli immune serum.

Number of Strains Employed for Absorption.	Agglutination with Serum Employed.	Dilution of Serum Employed for Absorption.	Time.	Temperature.	Strain Employed for Testing Unabsorbed and Absorbed Sera.	Agglutination with Unabsorbed Serum.	Agglutination with Absorbed Serum.	Percentage of Agglutinins lost by Absorption.
B. Coli I.	1–1,000	1–250	2 hrs.	37° C.	B. Coli I.	1–1,000 C.C.	± 1–250	75%
" " "	1–2,500	"	" "	"	" " "	1–2,500 I.C.	± 1–250	90%
B. Typhosus III.	1–10	1–250	2 hrs.	37° C.	B. Coli I.	1–1,000 C.C.	1–1,000	0%
" " "	"	"	" "	"	" " "	1–2,500 I.C.	"	60%
L.Y. 8 II.	Spontaneous agglutination.	1–250	2 hrs.	37° C.	B. Coli I.	1–1,000 C.C.	1–1,000	0%
" " "	Spontaneous agglutination.	"	" "	"	" " "	1–2,500 I.C.	"	60%

We are inclined to believe, however, that the figures indicating the loss of agglutinins due to centrifugalization are too high. They are obtained when the serum dilutions employed for the agglutination tests are graded in the usual manner. They represent, therefore, figures which would be obtained in practice but do not represent the actual loss which has occurred. These tables also show that the alteration referred to is not peculiar to meningococcus immune sera, but also occur with gonococcus and bacterium coli immune sera.

Before leaving this subject, we may stop to consider the fate of the agglutinins which disappear in the process of centrifugalization. In view of the inconstancy of this phenomenon, it is obvious that only variable factors in this part of the experiment deserve consideration. During the process of centrifugalization we find that the temperature of the fluid is raised. This rise, however, never exceeds 35° C., which is insufficient to appreciably alter the agglutinins. Moreover, the degree of temperature obtained in the instrument employed is fairly constant.

Experiments undertaken to determine whether an actual sedimentation of agglutinins occurs yielded negative results. While the titer of the surface fluid appeared at times to be slightly lower than that of the most dependent portion of the fluid, the differences were so slight as to come within the limits of experimental error. These experiments also excluded the possibility that the agglutinins were drawn down mechanically by small particles suspended in the serum. We were therefore led to the assumption that an actual destruction or alteration of the agglutinin molecule was brought about by centrifugalization.

The only variable factor in these experiments which might be responsible for this change and one, moreover, which cannot be wholly controlled, is the vibratory influence of the centrifuge. That differences in this respect occurred could not be established by the sense of touch, but was indicated by distinct variations in the tone emanating from our instrument while in motion. That the change in the agglutinin molecule is due to the uniform vibrations of a well-balanced centrifuge while in motion seems improbable. Such vibrations are constantly present and their number is fairly uniform, whereas the destructive action of centrifugalization on agglutinins is not always apparent. In order to determine the influence of uniform vibrations on the agglutinin molecule, we placed immune sera on top of rapidly running dynamos for eight to ten hours without causing any reduction in their agglutinating values. We assumed, therefore, that uniform vibrations are not responsible for these alterations in the agglutinin molecule, and were led to conclude that the discordant vibrations occurring when the instrument is improperly balanced, are responsible for the changes noted. It is quite conceivable that such vibrations are capable of causing a disruption of the agglutinin molecule and thus alter its properties.

In support of this view, we may add that since we have acquired a new instrument and adopted special precautions, such as the selection of centrifuge tubes identical in weight, a similar phenomenon has rarely been observed. Recently, however, similar changes have again occurred, although the usual precautions for a proper balancing of the instrument were observed. By taking the instrument apart and cleansing it, we noticed a distinct improvement in its action and simultaneously a disappearance of the discordant results under discussion.

To eliminate this source of error it is, therefore, necessary to employ a centrifuge control specimen.

ABSORPTION TESTS.

In the course of our investigations we have performed a large number of absorption experiments, all of which yielded results in conformity with the well-known facts established in connection with other bacteria, namely, that specific agglutinins are absorbed only by the members belonging to the same species and never by organisms belonging to another species. Since we have found no exceptions to this rule it seems unnecessary to record more than a few examples which suffice to establish the points we wish to make.

Table XXVI. is designed to show the results obtained with a meningococcus immune serum (M. 250 — rabbit No. 16) extracted with meningococcus strains of varying agglutinability.

TABLE No. XXVI.

Absorption of meningococcus immune serum with meningococcus strains of varying agglutinability.

Number of Strains Employed for Absorption.	Agglutination with Serum Employed for Absorption.	Dilution of Serum Employed.	Time.	Temperature.	Strain Employed for Testing Unabsorbed and Absorbed Sera.	Agglutination with Unabsorbed Serum.	Agglutination with Absorbed Serum.	Percentage of Agglutinins Lost by Absorption.
M. 250	1–5,000	1–250	2 hrs.	37° C.	M. 250	1–5,000	1–250	95%
" 250	1–4,000	"	" "	"	" "	1–4,000	1–500	87.5%
" 2451	1–500	"	" "	"	" "	1–6,000	1–3,000	50%
" 11	1–250	"	" "	"	" "	1–4,000	1–2,000	50%
" 5	1–100	"	" "	"	" "	1–6,000	1–5,000	16.66 + %
" 107	1–50	"	" "	"	" "	1–5,000	1–2,500	50%
" 12	1–25	"	" "	"	" "	1–6,000	1–4,000	33.33 + %
" 9	1–25	"	" "	"	" "	1–7,500	1–5,000	33.33 + %
" 8H	1–25	"	" "	"	" "	1–6,000	1–5,000	16.66 + %
" 71	1–10	"	" "	"	" "	1–7,000	1–6,000	15%

A review of these results shows that strains which are more or less inagglutinable exhibit reduced absorptive capacities. We may conclude, therefore, that an alteration of both the functional and haptophore groups comprising the agglutinable molecule has occurred. A comparison of the figures indicating the degree of agglutinability with those recording the absorptive capacities fails to reveal the existence of any definite relationship between absorptive capacities and agglutinability. For example, strain M. 2451 absorbs about the same number of agglutinins as does strain M. 107, yet the latter strain is practically inagglutinable, whereas the former responds to the action of the same serum in dilutions of 1–500. A comparison of the results obtained with strain M. 5 and strain M. 12 reveals similar discrepancies. We are, therefore, not warranted in assuming that the two components, which it is assumed make up the agglutinable molecule, are equally susceptible to external influences.

This experiment includes five strains which do not respond to the action of a powerful immune serum in dilutions above 1–50, yet absorption tests established their identity with other very agglutinable meningococcus strains.

The following table (XXVII.) shows the results obtained with a meningococcus immune serum extracted with pseudomeningococcus strains and two meningococcus strains.

TABLE NO. XXVII.

Number of Strains Employed for Absorption.	Agglutination with Serum Employed.	Dilution of Serum Employed for Absorption.	Time.	Temperature.	Strain Employed for Testing Unabsorbed and Absorbed Serum.	Agglutination with Unabsorbed Serum.	Agglutination with Absorbed Serum.	Percentage of Agglutinins Lost by Absorption.
M. 250	1–500	1–12.5	2 hrs.	37° C.	M. 250	1–500	1–50	70%
" 252	1–50	"	" "	"	" "	"	1–250	50%
P.M. 1	1–25	"	" "	"	" "	"	1–500	0%
" 2	O 1–25	"	" "	"	" "	"	"	0%
" 3	O 1–25	"	" "	"	" "	"	"	0%
" 4	1–25	"	" "	"	" "	"	"	0%
" 5	O 1–25	"	" "	"	" "	"	"	0%

This experiment shows that pseudomeningococcus strains are incapable of abstracting specific agglutinins from a meningococcus immune serum.

Table XXVIII. is inserted to show the results obtained when a pseudomeningococcus serum produced by injections of strain P.M. 2 was absorbed with pseudomeningococcus strains and meningococcus strains. After extraction the serum was retested with the homologous strain and meningococcus strain M. 250.

TABLE No. XXVIII.

Number of Strains Employed for Absorption.	Agglutination with Serum Employed.	Dilution of Serum Employed for Absorption.	Time.	Temperature.	Strain Employed for Testing Unabsorbed and Absorbed Serum.	Agglutination with Unabsorbed Serum.	Agglutination with Absorbed Serum.	Percentage of Agglutinins Lost by Absorption.
P.M. 2	1-500	1-12.5	2 hrs.	37° C.	P.M. 2	1-500	1-50	90%
" "	"	"	" "	"	M. 250	1-100	1-100	0%
" 1	± 1-25	"	" "	"	P.M. 2	1-500	1-500	0%
" "	"	"	" "	"	M. 250	1-100	1-100	0%
" 3	± 1-25	"	" "	"	P.M. 2	1-500	1-500	0%
" "	"	"	" "	"	M. 250	1-100	1-100	0%
" 4	O 1-25	"	" "	"	P.M. 2	1-500	1-500	0%
" "	"	"	" "	"	M. 250	1-100	1-100	0%
" 5	O 1-25	"	" "	"	P.M. 2	1-500	1-500	0%
" "	"	"	" "	"	M. 250	1-100	1-100	0%
M. 250	1-100	"	" "	"	P.M. 2	1-500	1-500	0%
" "	"	"	" "	"	M. 250	1-100	± 1-25	90%
" 252	O 1-25	"	" "	"	P.M. 2	1-500	1-500	0%
" "	"	"	" "	"	M. 250	1-100	1-25	75%
" 101	O 1-25	"	" "	"	P.M. 2	1-500	1-500	0%
" "	"	"	" "	"	M. 250	1-100	1-25	75%

± = Partial reaction.

In reviewing this table, the reader will note that the homologous strain alone is capable of removing specific agglutinins from this serum, and that the agglutinins acting on the meningococcus strain M. 250 are removed only by meningococcus strains. It follows, therefore, that neither a specific nor a group relationship can be established by these means between the pseudomeningococcus employed for the production of this serum and the meningococcus. The experiment also raises a doubt as to the identity of the various strains which we have grouped under the heading of pseudomeningococcus.

The tables which follow serve to record the results of absorption tests undertaken with the remaining groups of Gram negative cocci. A review of these tables clearly shows that absorption tests suffice to distinguish the various groups from each other and from the genuine meningococcus. They also show, however, that not all the members which we have placed in one group on account of their cultural and biochemical properties are identical when judged by absorption tests. In so far as our experience goes, this applies, however, only to the chromogenic Gram negative cocci and to pseudomeningococci. As we have already stated, Torrey's investigations show that agglutination and absorption tests fail to establish the identity of all the members which we have included under the heading "gonococcus."

Finally, an explanation is required for our failure to investigate the absorptive capacities of the strains belonging to the chromogenic group II. in the presence of the corresponding immune serum, and the influence which group I. and III. may exert on such a serum. At the time we had planned to undertake such experiments we found that none of the strains belonging to this group could be converted into stable suspensions in salt solutions. We were, therefore, not in possession of a strain which would serve to establish the number of agglutinins abstracted by a given strain. Subsequent attempts to produce a satisfactory immune serum also failed. The strains employed for this purpose were extremely toxic for rabbits. None of the animals remained alive long enough to yield a serum sufficiently powerful to serve for this purpose.

TABLE No. XXIX.

Absorption of meningococcus immune serum with a micrococcus catarrhalis, a gonococcus, and chromogenic groups I. and II. strains.

Number of Strains Employed for Absorption.	Agglutination with Serum Employed.	Dilution of Serum Employed for Absorption.	Time.	Temperature.	Strain Employed for Testing Unabsorbed and Absorbed Sera.	Agglutination with Unabsorbed Serum.	Agglutination with Absorbed Serum.	Percentage of loss of Agglutinins by Absorption.
M.C. 45	Spontaneous agglutination.	1-250	2 hrs.	37°	M. 250	1-6,000	1-6,000	0%
G. 7	1-500	"	" "	"	" "	1-7,000	1-7,000	0%
L.Y. 2 I.	1-10	"	" "	"	" "	1-6,000	1-6,000	0%
" 8 II.	Spontaneous agglutination.	"	" "	"	" "	1-7,500	1-7,500	0%

TABLE No. XXX.

Absorption of gonococcus immune serum with gonococcus, meningococcus, and micrococcus catarrhalis strains and a strain from chromogenic group I.

Number of Strains Employed for Absorption.	Agglutination with Serum Employed.	Dilution of Serum Employed for Absorption.	Time.	Temperature.	Strain Employed for Testing Unabsorbed and Absorbed Sera.	Agglutination with Unabsorbed Serum.	Agglutination with Absorbed Serum.	Percentage of Loss of Agglutinins by Absorption.
Gonococcus G. 7	1-5,000	1-250	2 hrs.	37° C.	G. 7	1-5,000	1-500	90%
Meningococcus M. 250	1-250	"	" "	"	" "	"	1-5,000	0%
Mic. Catarrhalis M.C. 55	Spontaneous agglutination.	"	" "	"	" "	"	"	0%
Chromogenic L.Y. 1 I.	± 1-10	"	" "	"	" "	"	"	0%
Gonococcus G. 7	1-2,000	"	" "	"	" "	1-2,000	1-500	75%
G. 16	1-250	"	" "	"	" "	"	1-1,000	50%
Meningococcus M. 250	1-50	"	" "	"	" "	"	1-2,000	0%

TABLE NO. XXXI.

Absorption of micrococcus catarrhalis immune serum with micrococcus catarrhalis and meningococcus strains.

Number of Strains Employed for Absorption.	Agglutination with Serum Employed.	Dilution of Serum Employed for Absorption.	Time.	Temperature.	Strain Employed for Testing Unabsorbed and Absorbed Sera.	Agglutination with Unabsorbed Serum.	Agglutination with Absorbed Serum.	Percentage of loss of Agglutinins by Absorption.
Micrococcus Catarrhalis. M.C. 50	Spontaneous agglutination.	1-25	2 hrs.	37° C.	M.C. 22 A.	1-1,000	1-100	90%
M.C. 22 A.	1-1,000	"	" "	"	" "	"	O 1-50	100%
M.C. 24	1-500	"	" "	"	" "	"	O 1-25	100%
Meningococcus M. 83	± 1-50	"	" "	"	" "	"	1-1,000	0%

TABLE NO. XXXII.

Absorption of chromogenic group I. Immune serum with two members of this group, a member of chromogenic group III., a genococcus and a meningococcus strain.

Number of Strains Employed for Absorption.	Agglutination with Serum Employed.	Dilution of Serum Employed for Absorption.	Time.	Temperature.	Strain Employed for Testing Unabsorbed and Absorbed Serum.	Agglutination with Unabsorbed Serum.	Agglutination with Absorbed Serum.	Percentage of Agglutinins lost by Absorption.
L.Y. 1 I.	1-1,000	1-100	2 hrs.	37° C.	L.Y. 1 I.	1-1,000	1-100	90%
" 3 I.	1-25	"	" "	"	" "	1-2,500	1-500	80%
" 13 III.	± 1-10	"	" "	"	" "	"	1-2,500	0%
G. 10	1-50	"	" "	"	" "	"	1-2,500	0%
M. 250	1-100	"	" "	"	" "	1-1,000	1-1,000	0%

± = Partial reaction.

TABLE NO. XXXIII.

Absorption of immune serum produced by L.Y. 12 chromogenic group III. with strains from chromogenic groups I., II., and III., a meningococcus and a gonococcus strain.

Number of Strains Employed for Absorption.	Agglutination with Serum Employed.	Dilution of Serum Employed for Absorption.	Time.	Temperature.	Strain Employed for Testing Unabsorbed and Absorbed Serum.	Agglutination with Unabsorbed Serum.	Agglutination with Absorbed Serum.	Percentage of Loss of Agglutinins by Absorption.	
L.Y. 12 III.	1-7,500	1-100	2 hrs.	37° C.	L.Y. 12 III.	1-7,500	1-2,500	66⅔%	Chromogenic group III.
" 15 "	1-500	"	" "	"	" " "	1-7,500	1-5,000	33⅓%	" " "
" 13 "	1-250	"	" "	"	" 13 "	1-250	O1-100	100%	" " "
L.Y. 1 I.	1-10	1-100	2 hrs.	37° C.	L.Y. 13 III.	1-250	1-250	0%	Chromogenic group I.
L.Y. 8 II.	Spontaneous agglutination.	1-250	2 hrs.	37° C.	L.Y. 12 III.	1-7,500	1-7,500	0%	Chromogenic group II.
M. 250	1-50	1-100	2 hrs.	37° C.	L.Y. 12 III.	1-7,500	1-7,500	0%	Meningococcus.
" "	"	"	" "	"	" 13 "	1-250	1-250	0%	"
G. 7	1-250	1-100	2 hrs.	37° C.	L.Y. 12 III.	1-7,500	1-7,500	0%	Gonococcus.

The following table shows that the agglutinins in a meningococcus immune serum acting on the diplococcus crassus are not identical with those determining the clumping of the meningococcus. Extraction of this serum with the homologous meningococcus strain fails to remove the substances acting on the diplococcus crassus, and absorption of this serum with the diplococcus crassus does not reduce its agglutinating value for the meningococcus.

TABLE No. XXXIV.

Absorption of meningococcus immune serum with a meningococcus and a diplococcus crassus strain.

Number of Strains Employed for Absorption.	Agglutination with Serum Employed.	Dilution of Serum Employed for Absorption.	Time.	Temperature.	Strain Employed for Testing Unabsorbed and Absorbed Serum.	Agglutination with Unabsorbed Serum.	Agglutination with Absorbed Serum.	Percentage of Agglutinins Lost by Absorption.
M. 250	1–2,500	1–12.5	2 hrs.	37° C.	M. 250	1–2,500	1–250	90%
" "	"	"	" "	"	D. Crassus	1–12.5	1–12.5	0%
D. Crassus	1–12.5	"	" "	"	M. 250	1–2,500	1–2,500	0%
" "	"	"	" "	"	D. Crassus	1–12.5	O 1–12.5	100%

GENERAL SUMMARY AND CONCLUSIONS CONCERNING AGGLUTINATION AND ABSORPTION TESTS.

In reviewing the results obtained in connection with this phase of our work, the following facts deserve to be emphasized:

1. The unusual instability of the agglutinable properties of the meningococcus and the marked variations in the degree of agglutinability exhibited by different meningococcus strains in the presence of the same immune serum deprives these tests of much of their diagnostic value. Approximately forty per cent of the meningococcus strains studied were relatively inagglutinable. Recently isolated strains were on the whole less sensitive to the action of agglutinins than were older strains.

2. The presence of serum in the culture medium exerted a deleterious influence on the agglutinable properties of meningococcus strains.

3. Passage through plain or glucose bouillon rendered some of the inagglutinable strains more sensitive to the action of agglutinins, whereas others were not influenced or were unfavorably affected by this treatment.

4. Since spontaneous agglutination of these organisms may occur in various serum dilutions and be absent in the normal salt solution control suspension, parallel tests with normal serum should be undertaken in each instance.

5. Temperature exerts no marked influence on the end results of these reactions. While incubator temperature and a temperature of 55° C. accelerated the reaction, the values obtained after the lapse of twenty-four hours were not materially higher than those obtained at room temperature. We have not succeeded in rendering an inagglutinable strain more sensitive by exposing the tests to a temperature of 55° C.

6. For the completion of the agglutination reaction with these organisms at least twenty-four hours are required.

7. Centrifugalization accelerates the clumping and sedimentation of agglutinable strains suspended in the corresponding immune serum.

8. In comparing the rate of the reaction occurring when an inagglutinable meningococcus strain was exposed to the action of the corresponding immune serum with the velocity of the reaction occurring when a member of another group was exposed to the action of the same serum, it was observed that the meningococcus strains responded more promptly to the action of the immune serum than did the heterologous strains, although after the lapse of twenty-four hours both strains were clumped to the same extent. These differences in the rate of the reaction suggest difference in the avidity with which the substances interacting combine, or suggest fundamental differences in the substances participating in such reactions.

9. While the meningococcus, as a rule, fails to respond to the action of normal rabbits' serum diluted above 1–25,

certain strains appear to be unusually sensitive to the action of such sera. A few strains were clumped in normal serum in dilutions of 1–250.

10. The agglutinogenic capacities of the meningococcus are very considerable. In the course of our work we have produced meningococcus immune sera which agglutinated the homologous strain in serum dilutions of 1–25,000. The agglutinogenic capacities of the inagglutinable strains though limited were sufficient to produce immune sera of moderate potency when tested with agglutinable strains. Toward the homologous inagglutinable strains they were ineffective. The failure to respond to the action of an immune serum on the part of certain strains is, therefore, not due to any peculiarities of the serum employed, but to reduced clumping capacities of the organisms.

11. A definite relationship between agglutinogenic properties and agglutinin absorptive capacities as a rule appears to exist.

12. Failure to combine with agglutinins in a test-tube experiment does not signify a total loss of agglutinogenic properties.

13. What has been said concerning the behavior of the meningococcus toward the corresponding immune serum applies also to the behavior of the other Gram negative cocci toward their homologous sera.

14. When properly controlled, agglutination tests serve to differentiate the various groups from each other, providing the strains tested are sufficiently agglutinable. Diagnostic difficulties may arise in connection with certain gonococcus strains. These are unusually sensitive to the action of normal and of group agglutinins and may yield higher values in the presence of a meningococcus immune serum than moderately agglutinable meningococcus strains.

15. Absorption tests served to differentiate the various groups of Gram negative cocci from each other and to establish the identity of the agglutinable and inagglutinable meningococcus strains. One genuine meningococcus strain,

however, failed to absorb specific agglutinins from an homologous immune serum.

16. Meningococcus strains which were more or less inagglutinable exhibited reduced absorptive capacities, but a definite relationship between the binding capacities of such strains and their agglutinability could not be established.

17. These tests also revealed the existence of a group relationship between the meningococcus and gonococcus.

18. Judged by the results of absorption tests, the pseudomeningococcus group and the chromogenic group I. require a further subdivision. At least two varieties are represented in each of these groups.

19. A relationship of the Jaeger coccus and the diplococcus crassus to the meningococcus could not be established by these tests.

20. Under certain conditions, centrifugalization of an immune serum causes a reduction of its agglutinating value. This alteration of the agglutinins we have attributed to the influence of discordant vibrations of the centrifuge. Since this cannot be wholly controlled, the necessity for a centrifuge control specimen is evident, if we clarify an absorbed serum by these means.

AGGLUTINATION TESTS WITH HUMAN SERA.

From what has been said in the preceding sections concerning the erratic behavior of the meningococcus toward artificially produced immune sera, it is evident that the diagnosis of epidemic cerebro-spinal meningitis by means of agglutination tests is surrounded by many difficulties.

The values obtained with a given serum are in large part determined by the strain employed for the tests. In the course of our work we have encountered two sera derived from cases of meningitis which agglutinated one strain in dilutions of 1–400, yet the values obtained with four other strains did not exceed 1–50. While none of these strains was agglutinated by normal serum or serum derived from patients suffering from other diseases, tests undertaken one week later with sera derived from two cases of typhoid, one of lobar pneumonia, and one of tuberculous peritonitis, showed that the transplanted strains had become unusually sensitive to the action of non-specific agglutinins. The serum from one of the typhoid patients agglutinated the most agglutinable strain in dilutions of 1–100, and the four other strains to the same extent (1–50) as the serum from two meningitis cases previously tested. It is evident, therefore, that a standard even for a given strain cannot be established which could be accepted as of diagnostic value and that every test should be controlled by parallel tests with normal serum. The difficulty, however, still remains, that certain strains may become inagglutinable in the course of cultivation and thus yield erroneous results. The practical difficulties surrounding these tests are, therefore, considerable, yet in cases which come under observation late in the disease this may be the only laboratory means of arriving at a diagnosis. For example, in two chronic cases in which a diagnosis could not be established by an examination of the spinal fluid, we were able to enforce the clinical diagnosis by means of agglutination tests.

To overcome the difficulties indicated above, von Lingelsheim resorted to the use of killed meningococcus cultures, and claims to have obtained reliable results by these means. Among five hundred and ninety-three samples of serum from meningitis cases this observer obtained positive results in two hundred and eighteen instances. A complete reaction in serum dilutions of 1–25 was accepted by von Lingelsheim as positive.

BACTERIOLOGICAL DIAGNOSIS OF EPIDEMIC CEREBRO–SPINAL MENINGITIS.

A positive diagnosis of this disease during life rests on the demonstration of the causal agent in the cerebro-spinal fluid withdrawn by lumbar puncture. While this, as a rule, presents no difficulties in recent cases, such a demonstration may be impossible in cases coming under observation late in the disease, either because the organisms are no longer present or because it may be impossible to obtain a sample of this fluid by lumbar puncture. Under these conditions it may be possible to establish a diagnosis by means of agglutination tests, although, as we have already indicated, a positive reaction, even though obtained with a much diluted serum, cannot be accepted without reserve unless controlled by parallel tests with normal serum.

As illustrating the number of times a diagnosis based on an examination of the spinal fluid may be expected, the following statistics are cited:

Among two hundred and ten cases coinciding clinically with this form of meningitis, a bacteriological examination of the spinal fluid served to establish a positive diagnosis in one hundred and ninety-four instances (92.4 per cent).

If we tabulate these cases in weekly periods according to the stage of the disease when the examination of the lumbar puncture fluid was undertaken, we obtain the following figures:

	Results.					
	Film Preparations.		Cultures.		Combined Method.	
	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
134 cases examined during the 1st week of the disease......	100	20	94	16	124	10
21 cases examined during the 2d week of the disease.	13	1	16	3	20	1 (12th day).
13 cases examined during the 3d week of the disease.......	8	1	11	0	13	0
9 cases examined during the 4th week of the disease......	6	2	7	1	8	1 (23d day).
4 cases examined during the 5th week of the disease......	1	0	3	0	4	0
1 case examined during the 6th week of the disease..........	1	0	1	0	1 (36th day).	0
1 case examined during the 8th week of the disease..........	1	0	1	0	1 (50th day).	0
1 case examined during the 11th week of the disease.....	1	0	1	0	1 (74th day).	0
26 cases in which duration of disease was doubtful	10	6	18	5	22	4
	141	30	152	25	194	16

A review of this table shows that a microscopic examination of the spinal fluid from one hundred and seventy-one cases yielded one hundred and forty-one positive results (82.46 per cent) and a cultural examination undertaken in one hundred and seventy-seven cases furnished positive results in one hundred and fifty-two instances (85.88 per cent). An examination of a single specimen of spinal fluid did not suffice to establish a positive diagnosis in quite a number of instances. This was particularly true when we relied for our diagnosis upon the isolation of the organism in cultures. These failures we are inclined to attribute in part to the fact that approximately one-fourth of the cultures could not be placed immediately under suitable conditions for growth. The importance of this factor is clearly shown if we compare the results obtained by Bettencourt and Franca,[7] who apparently worked under the most favorable conditions, with those obtained by von Lingelsheim.[8] The former observers were able to isolate the meningococcus in every instance from the spinal fluid derived from two hundred and seventy-one cases of epidemic cerebro-spinal meningitis (one hundred per cent). Von Lingelsheim,[9] on the other hand, working with material which could not be cultivated immediately, failed to isolate the meningococcus in approximately forty-two per cent of three hundred and eight samples of spinal fluid, while in a series of one hundred and thirteen specimens which could be cultivated more promptly, he succeeded in obtaining positive results in seventy-eight and three-tenths per cent of the cases. We may infer, therefore, that the cultivation of the spinal fluid immediately after its withdrawal is of considerable importance.

Of the sixteen cases in our series which were negative throughout, twelve were examined but once, four were examined twice. It will be noticed that ten of these cases were examined during the first week of the disease, in four the duration was doubtful and the two remaining cases were examined on the twelfth and twenty-third day of the disease respectively. In the absence of positive bacteriological findings the nature of these cases remains doubtful.

While we have obtained positive results in very chronic cases, the results of other observers show that the chances of establishing a positive diagnosis diminish with the duration of the disease. Repeated examinations of some of our cases showed that the number of organisms in the spinal fluid tend to diminish as the disease advances, which is in accord with observations made by others.

It remains to be mentioned in this connection that with two exceptions, which will be considered later, the meningococcus appeared to be the only microörganism concerned in the production of the disease. While contamination of our cultures with the staphylococcus albus was not an infrequent occurrence, an examination of a second sample of spinal fluid from the same case revealed the meningococcus in pure culture. In one case the streptococcus appeared to be associated with the meningococcus, but our failure to recover this organism from a second specimen of the spinal fluid suggests accidental contamination of the first sample withdrawn.

Owing to the great importance of arriving at an early diagnosis in this disease, it may be profitable to consider what bacteriological data are necessary to establish this point beyond a reasonable doubt. Is it essential to isolate the causative factor in cultures and expose it to the various tests considered in the preceding sections, or would the demonstration in film preparations made from the spinal fluid, of a Gram negative coccus, having the morphological and staining characters of the meningococcus, suffice to establish a positive diagnosis? The answer to this question is determined by the number of times Gram negative cocci have been observed and isolated from the cerebro-spinal fluid, which, as shown by subsequent tests, were not genuine meningococci.

In our series of cases we have isolated Gram negative cocci from the spinal fluid one hundred and fifty-two times. With few exceptions these strains were submitted to the various tests indicated in the preceding sections. All of the strains thus tested proved to be genuine meningococci. In one instance, however, we recovered a Gram negative coccus from a culture flask inoculated with cerebro-spinal fluid which did not conform to the meningococcus in all respects. A film preparation from this sample of spinal fluid was not examined. This flask was not placed in the incubator immediately, and it had to be transported from Hudson Street Hospital, some two miles from the laboratory. These facts, and the further fact that the genuine meningococcus was subsequently isolated from this case, leads us to infer that in this instance we were dealing with a contamination.

Flügge[14] examined one hundred and forty samples of spinal fluid derived from suspected cases of meningitis. Among these he encountered Gram negative cocci, which were not meningococci, in five instances. Since Flügge's material was sent to him from distant parts, and the fluid withdrawn by men who were probably not wholly familiar with this procedure, it seems probable that the organisms isolated represented contaminations.

Von Lingelsheim[9] also encountered Gram negative cocci not identical with meningococci in cultures made from the lumbar puncture fluid. The Gram negative organisms, other than meningococci, recovered by this observer from three hundred and eight samples of this fluid were the diplococcus mucosus in one case, the diplococcus pharyngis flavus II. in one case, and in a number of instances (exact number not stated) the micrococcus cinereus. The diplococcus crassus, which was usually Gram positive in films made from the exudate, was encountered rather frequently by this observer. None of the organisms enumerated were found within leucocytes.

The only frankly Gram negative coccus which appeared in the spinal fluid more than once in von Lingelsheim's cases was the micrococcus cinereus, but a definite relationship of this organism to the cases furnishing the spinal fluid was not established. Since the materials examined by von Lingelsheim[9] were for the most part shipped from parts some distance removed from the laboratory, it seems probable that these organisms also represent contaminations.

Finally, a review of the literature reveals a number of cases of meningitis which were attributed to the gonococcus. Such cases have been reported by Engel-Reimers,[55] Polozoff,[56] D'Amato,[57] Hoening,[58] Prochaska,[59] and Josselin de Jong.[14] Among these, only the cases reported by Prochaska and de Jong were examined bacteriologically. These observers isolated an organism from the spinal fluid, which in its morphological, cultural, and staining characteristics resembled the gonococcus. When we consider, however, that the meningococcus and gonococcus cannot be positively differentiated in cultures nor in film preparations, more convincing proof is required to establish the gonococcic nature of the organisms encountered by these observers.

In conclusion, therefore, we may say that the occurrence in a lumbar puncture fluid of Gram negative cocci situated within pus cells suffices to establish a diagnosis of epidemic cerebro-spinal meningitis, and that the occurrence of Gram negative cocci occupying an extracellular position furnishes a reasonably certain basis for such a diagnosis providing the examination of the fluid is undertaken immediately after its withdrawal.

From what has already been said, it is evident that a complete bacteriological investigation is necessary for the identification of Gram negative cocci recovered from parts removed from the central nervous system.

The diagnosis of epidemic cerebro-spinal meningitis at autopsy also rests upon the demonstration of the meningococcus in the parts involved. In a series of thirty-three cases of epidemic meningitis, which were examined at autopsy, the meningococcus was demonstrated in film preparations made from the meningeal exudate in twenty-nine instances. It was isolated from the meninges in twenty-three cases. In two cases the organisms failed to grow in cultures, although present in the exudate. In both instances the disease was of short duration (twenty-one and thirty-one hours respectively) and the autopsy was undertaken within fifteen hours after death. In five cases the cultural investigation was omitted, the organism in four of these having been recovered from the spinal fluid withdrawn during life. The meningococcus was demonstrated in films in all of these cases.

Finally, in three instances, the organisms could not be demonstrated either in film preparations or in cultures made from the meningeal exudate. These were chronic cases extending over a period of eleven days, thirty-four days, and seventy-nine days respectively. In the first case, dying after the lapse of eleven days, the autopsy could not be performed until forty-eight hours had elapsed, which may explain our failure to obtain the organism in cultures. In the second and third cases, the autopsy was performed within seventeen hours after death. The blood serum taken from the third case at autopsy agglutinated a meningococcus strain in dilutions of 1–500, whereas blood serum taken from a patient suffering from chronic nephritis did not agglutinate this strain in serum dilutions above 1–250. It seems probable, therefore, that the reaction obtained in this instance was specific. By the combined cultural and microscopic methods a positive diagnosis of this disease was, therefore, established in thirty out of thirty-three cases examined at autopsy.

Before leaving this subject the question of mixed infections may be briefly considered.

While Bettencourt and Franca[7] isolated the meningococcus in pure culture in two hundred and seventy-one cases examined, von Lingelsheim[9] frequently encountered a number of organisms in the lumbar puncture fluid. In one hundred and ninety-eight samples of spinal fluid, the latter observer isolated the meningococcus in pure culture in one hundred and eighty instances. In the remaining samples he found, in addition, the staphylococcus in twelve and the diplococcus crassus in six. He is inclined to attribute the presence of the former organism to a contamination of the fluid during its withdrawal. In thirty-one cases examined at autopsy shortly after death, this observer found the meningococcus in every instance, and in five of these it was associated with the diplococcus crassus. Since these latter organisms were found in four out of five fulminant cases, this observer attributed the rapid course of the disease to the fact that a mixed infection existed. In a later communication, however, he is inclined to regard these associated organisms as secondary invaders only. In our own series only two instances of mixed infection of the meninges occurred among two hundred and ten cases examined. In both instances the pneumococcus was found associated with the meningococcus. We may infer, therefore, that mixed infection of the meninges in this disease is rare. Even if we accept von Lingelsheim's fulminant cases as instances of mixed infection with the diplococcus crassus, we would have only thirteen cases of mixed infection among a total of six hundred and seventy-nine cases, or approximately two per cent.

Judging from the literature, the association of the diplococcus intracellularis with the tubercle bacillus is by no means uncommon. An analysis of the cases recorded, however, shows that in the majority of instances the organisms isolated from the spinal fluid were not identical with the Weichselbaum coccus. Holdheim[60] reported the first case of this kind and Heubner[61] followed with two others. In these, the organisms could not be demonstrated in film preparations from the spinal fluid, and the organisms isolated in cultures corresponded to the so-called Jaeger type of the meningococcus. Another instance of mixed infection with the tubercle bacillus was reported by Hunter and Nuttall.[62] Here again the organisms were not found in the spinal fluid immediately after its withdrawal. After twenty-four hours' incubation the fluid showed cocci which were isolated in cultures. They formed short chains, were surrounded by distinct capsules, and grew at room temperature. In these respects the organism differs from the Weichselbaum coccus. Lenhartz,[63] Frohmann,[64] and Netter[65] also reported instances of tuberculous meningitis in which an organism believed to be the meningococcus was isolated from the spinal fluid. These observers, however, were unable to demonstrate the presence of these organisms in film preparations, and the observations were made at a time when considerable confusion existed concerning the characteristics of the diplococcus intracellularis.

On the other hand, Libman[66] records bacteriological examinations of the spinal fluid in forty-four cases of tuberculous meningitis without finding a single instance of mixed infection with the meningococcus.

In a series of twenty-five cases of tuberculous meningitis we also failed to find a single instance of such a mixed infection.

When we bear in mind that New York City is rarely wholly free from cases of epidemic cerebro-spinal meningitis it is evident that the conditions for such a coincidence are particularly favorable, yet so far as we are aware an instance of mixed infection of the meninges with the tubercle bacillus and the meningococcus occurring in this city has not been recorded. We feel justified, therefore, in concluding that an association of the diplococcus intracellularis with the tubercle bacillus in the meninges is a rare occurrence.

Part II.

VIABILITY OF THE MENINGOCOCCUS.

This phase of the meningococcus problem has been the subject of many investigations, but the results obtained by earlier observers varied widely. While Weichselbaum,[1] Albrecht and Ghon,[6] Councilman, Mallory and Wright,[5] and Bettencourt and Franca[7] contended that the meningococcus was unusually sensitive to external influences, Jaeger,[4, 10] Germano,[67] and others arrived at opposite conclusions. These differences of opinion were due to the fact that the two groups of investigators were not working with the same species. Since this has been established beyond a doubt, and since it has been shown that the organisms employed by Jaeger and Germano were not the specific cause of epidemic cerebro-spinal meningitis, although occasionally encountered in connection with it, all subsequent observers have confirmed Weichselbaum's original observations bearing on this question. Among these later investigators the following deserve mention: Schottmüller,[68] Jochmann,[44] Manteufel,[69] von Lingelsheim,[9] Flügge,[54] Kolle and Wassermann,[46] and Kutscher.[70] Our own observations confirm in the main those made by other investigators working with the genuine meningococcus.

Viability on culture media. — We found that recently isolated strains died out rapidly on culture media. To insure the maintenance of such a strain, it is necessary to transfer the organism to new culture media daily. While many recently isolated strains can be recovered after forty-eight hours' incubation, we have encountered a number of strains which could not be carried beyond the second or third generation, although daily transplants were made. Fortunately, such hypersensitive strains are not numerous. Among two hundred strains isolated by us only three belonged to this group. These were all isolated from autopsy material and showed their incapacity to thrive well on artificial media on the original plates. Two died out in the third generation, while the third failed to grow after the first generation. The question arises whether these organisms were actually meningococci. Morphologically and culturally they were identical with the more viable meningococcus strains. Moreover, the fact that they were recovered from bodies in which a bacteriological diagnosis of epidemic cerebro-spinal meningitis had been established during life leaves little doubt as to their identity.

In transferring recent strains of this coccus it is important to employ a considerable amount of culture material and to maintain the growth on a medium equalling in point of richness that employed for the initial cultivation.

Under artificial cultivation the organism becomes more resistant. Serum media are no longer essential and the intervals for transplantation can be prolonged. With old cultures we have had little difficulty in maintaining growth by transplanting once a month, providing suitable provisions were made to prevent drying. It must be mentioned, however, that certain strains in constant use suddenly ceased to grow, and all attempts to recover the same from the preceding tube failed.

Considering the limited viability of young cultures, the longevity of old strains in culture media protected against drying and surrounded by a suitable temperature is rather remarkable.

The following table (No. XXXV.) shows that some strains grown on ascitic agar slants and protected against drying survived for two hundred and eighty-two days at incubator temperature, while three strains could be recovered after three hundred and thirty-three days had elapsed.

TABLE No. XXXV.

Viability of the meningococcus on suitable culture media when kept in a moist condition.

	Number of Strain.	Source.	Recovered.	
	M. 22 I.	Spinal fluid.	+ 282 days	6 of 8 strains recovered.
	" 22 II.	" "	+ " "	
	" 6 I.	" "	+ " "	
	" 50	" "	+ " "	
	" 35	" "	+ " "	
	" 44	Blood.	+ " "	
Same Case.	" 74	"	O " "	
	" 73	Spinal fluid.	O " "	
	M. 6 II.	Spinal fluid.	+ 305 days	1 of 2 strains recovered.
	" 22 I.	" "	O " "	
	M. 22 I.	Spinal fluid.	O 331 days	0 of 2 strains recovered.
	" 22 III.	" "	O " "	
	M. 22 II.	Spinal fluid.	+ 333 days,	3 of 3 strains recovered.
	" 6 I.	" "	+ " "	
	" 6 II.	" "	+ " "	

Further investigations carried out with these strains showed that they had lost none of their original cultural or biochemical characteristics. Films made from the culture tubes in question showed only an occasional coccus of normal shape and staining properties. The vast majority were represented by mere shadows which were scarcely colored; others were unusually large and densely stained. All were Gram negative. In order to determine whether these large individuals represented resistant or so-called permanent forms, desiccation experiments were undertaken. These showed, however, that the organisms in question were even slightly less resistant to drying than were many other meningococcus strains.

Influence of temperature. — Our observations concerning the influence of moist heat on meningococci are somewhat at variance with those of other observers. According to Bettencourt and Franca,[7] exposure to temperatures of 50° C. moist heat for five minutes and to 55° C. for three minutes suffices to destroy the meningococcus. According to the figures obtained by von Lingelsheim,[9] these organisms are somewhat more resistant. A temperature of 50° C. moist heat was tolerated by the meningococcus for one hour, and it required ten minutes to destroy this organism at 60° C. Our own figures approximate those of von Lingelsheim. Four of our strains resisted a temperature of 45° C. moist heat for three hours, and two were recovered after an exposure of two hours to 55° C. These experiments were carried out as follows:

A series of bouillon tubes were placed in a closed water bath. After the temperature had reached the desired grade, the tubes were inoculated with two standard loopfuls of a twenty-four-hour growth. At various intervals the tubes were removed and rapidly cooled to body temperature in cold water. Each tube now received ascitic fluid in the proportion of one to two, and was placed at incubator temperature.

The different results obtained by various observers are probably only in part due to difference in the methods employed. Differences in the age of the culture employed and individual differences among the strains may explain the discordant results recorded in the literature.

Influence of room temperature.—While none of our strains showed growth at a temperature below 25° C., many of them remained alive at room temperature for rather long periods. Kept in a dark room, nine strains grown on glucose agar lived for three weeks, six strains survived for four weeks, but none could be recovered after five weeks' exposure. These figures are also at variance with the results obtained by von Lingelsheim.[9] According to this observer, the meningococcus exposed to room temperature, as a rule, dies out within five days. Our results are, however, not comparable to those of von Lingelsheim,[9] since the temperature of our laboratory is considerably higher than normal room temperature. An approximate record of the temperature during the time our experiments were carried out shows that the temperature varied from 25° to 26° C. during the day.

Influence of ice-box temperature (8° C.). — Exposed to ice-box temperature, nine strains grown on glucose agar remained alive for three days, seven strains for four days, four strains for five days, and one strain for six days. None survived exposure to ice-box temperature for one week. These figures are in complete accord with those of von Lingelsheim[9] and of Kutscher.[28, 70] Bettencourt and Franca,[7] however, found that a number of their strains kept at ice-box temperature remained alive for one month.

While the results of different observers are not uniform, all are agreed that the temperature of the incubator is the most suitable for the maintenance of meningococcus cultures.

Influence of light. Diffuse daylight. — The tests designed to determine the influence of diffuse daylight were carried out in conjunction with those undertaken to determine the effects of room temperature. Exposed to diffuse daylight, nine cultures, representing different strains, remained alive five days, eight survived six days, and four could be recovered after seven days' exposure; none after eight days. In comparing these figures with the results obtained in cultures exposed to room temperature, but protected against diffuse daylight, the deleterious influence of diffuse light is apparent.

Direct sunlight. — The results yielded by these tests are rather surprising. Nine strains exposed to the direct rays of the sun during the month of August remained alive for eight hours, and eight strains could be recovered after nine hours' exposure. In this connection we may mention the results of parallel investigations undertaken by other observers. According to Bettencourt and Franca[7] (Portugal), two hours sufficed to destroy the meningococcus when exposed to direct sunlight; von Lingelsheim[9] (June — Berlin) found four to five hours necessary to accomplish this end and Kutscher[28, 70] (March — Berlin) found that eight to twelve hours were required to destroy them.

While these figures are not wholly comparable, they seem to show that the meningococcus is fairly resistant to sunlight.

Influence of desiccation. — It is generally accepted at the present time that the meningococcus does not resist drying to any extent. Most observers agree that twenty-four hours' desiccation on glass suffices to destroy the meningococcus. Our own experiences in this connection lead us to assume that they are slightly more resistant than is generally supposed.

Experiments bearing on this point were carried out in the following manner:

Each strain tested was grown on solid media. After twenty-four hours', forty-eight hours', and one week's incubation a portion of the growth was removed from the surface with a sterile swab and smeared over the inside of a series of sterile test-tubes, near the bottom. Care was taken to avoid moisture in extracting the material from the culture tubes. The film thus formed dried immediately. After twenty-four, forty-eight and seventy-two hours at room temperature had elapsed, the medium selected for the recovery of the organisms was poured into the several test-tubes and set aside at incubator temperature. Those showing growth were transferred to plates to establish the purity of the growth and for purposes of reidentification. For the recovery of the dried organisms three varieties of media were employed, all of which offered, as shown by control tests, favorable conditions for the growth of the organisms selected for these tests.

The results obtained are recorded in the following table, No. XXXVI.:

TABLE NO. XXXVI.

Viability of the meningococcus when dried on glass. Ten strains employed.

Duration of Drying.	Media Employed for Recovering Strains.	24-Hour Cultures.	48-Hour Cultures.	1-Week Cultures.
24 hours	Plain broth.	0 strains.	3 strains.	1 strains.
	Glucose "	1 "	2 "	1 "
	Ascitic "	3 "	4 "	3 "
48 hours	Plain broth.	0 strains.	1 strains.	0 strains.
	Glucose "	0 "	2 "	0 "
	Ascitic "	0 "	2 "	2 "
72 hours	Plain broth.	0 strains.	1 strains.	0 strains.
	Glucose "	0 "	1 "	0 "
	Ascitic "	0 "	3 "	1 "

A review of the preceding table shows that two factors are of importance in determining the results of such experiments, namely, the age of the growth and the medium used for the recovery of the dried organisms. It will be noted that organisms which were allowed to grow for forty-eight hours were more resistant to drying than were twenty-four hour or one week growths. Of the twenty-four-hour growths, only four strains out of ten tested survived desiccation for twenty-four hours, whereas of the same strains grown for forty-eight hours, nine were recovered after twenty-four hours' drying and five after seventy-two hours. The same strains grown for one week were slightly more resistant than twenty-four-hour cultures, though distinctly less so than forty-eight-hour growths.

Concerning the influences of the culture medium on the results of these experiments, it will be noted that ascitic broth gave the best results. The practical significance of these facts will be considered in a subsequent section.

PATHOGENICITY OF THE MENINGOCOCCUS FOR ANIMALS.

All observers are agreed that this organism enjoys only a moderate grade of pathogenicity for laboratory animals. It is, as a rule, incapable of producing disease in small animals when introduced subcutaneously. When injected intraperitoneally, it commonly causes death of mice and half-grown guinea-pigs, but usually fails to produce an acute illness in rabbits. With the exception of the monkey, and possibly the goat, a disease comparable to that occurring in man cannot be reproduced in animals by means of this organism.

Among the smaller animals, the white mouse, according to our observations, appears to be the most susceptible, which is in accord with the findings of Bettencourt and Franca. Other observers, von Lingelsheim and Leuchs,[71] Flexner,[72] and Kolle and Wasserman,[46] are inclined to accept half-grown guinea-pigs as the most favorable animals for experimental inoculation. Different races of white mice employed by various observers may explain such differences. The particular lot of white mice employed by us was perhaps slightly less resistant than is usual, since occasionally subcutaneous injections sufficed to cause the death of the animal. On the whole, however, they resisted subcutaneous injections. The statements which follow are based on observations made on, approximately, one hundred mice.

Concerning intraperitoneal injections, we can record that the majority of the strains tested produced a fatal disease in white mice when thus administered. Death, as a rule, occurred within twenty-four or forty-eight hours. The dose required to bring about this end varied considerably, not only for different strains but for the same strain employed on different days, which is in accord with the experiences of other observers. The M.L.D. established on one day did not correspond with the figures obtained on the following day with the same strain grown on the same lot of medium, indicating marked variations in the virulence of meningococcus strains. Moreover, great differences in the individual susceptibility of the same species of animals appear to exist. In a series of animals receiving graded doses of the same suspension it was impossible to predict which animal would succumb first or which was destined to recover. In several instances the animal receiving the largest dose recovered, whereas those receiving smaller doses died. We were unable to augment the virulence of a strain by repeated passage through mice.

The anatomical findings at autopsy were fairly constant. The intestines were usually distended and red in color. The peritoneum contained a small amount of a viscid exudate. In some instances the number of leucocytes present in this exudate was inconsiderable, whereas in others they were fairly abundant. The spleen was congested and, as a rule, slightly enlarged.

The bacteriological findings in mice dying of experimental inoculation with the meningococcus varied. In some, the peritoneum contained numerous meningococci, both extra and intracellular; in others the number of organisms was small and in a few instances could only be demonstrated by culture. As a rule, the organism could be isolated from the heart's blood.

The question as to the nature of the disease induced in mice by injections of the meningococcus is variously answered by different observers. While Weichselbaum[1] in his original communication assumed that the death of mice thus treated was due to a true infection, later investigators seem inclined to attribute the disease produced by experimental inoculation largely to intoxication.

The almost constant invasion of the blood stream and the fact that in certain mice an actual multiplication of the organisms in the peritoneum, judging from the number present in the exudate, seems to have occurred, strongly suggest that a true infection had taken place. Both these observations are, however, capable of another interpretation. Moreover, in other animals the bacteriological findings in the peritoneum would seem to indicate that a simple intoxication was the cause of death. The following experiments also suggest that the element of infection, *i.e.*, multiplication of the organisms within the animal body, is not an essential factor in the production of disease in animals. A series of mice was injected intraperitoneally with graded cases of a virulent meningococcus culture suspended in normal salt solution. A measured portion of this suspension was rapidly dried in vacuo over sulphuric acid. After three days' desiccation, which sufficed to kill all the organisms (cultural tests), enough sterilized distilled water was added to bring the mixture up to the volume of the original suspension. A second series of mice was injected with graded doses of this suspension. The result of this experiment showed that the dose of killed organisms required to bring about a fatal issue in mice was double that necessary to accomplish the same end with the living organism. It would seem, therefore, that the element of infection, in so far as this strain is concerned, is not an important factor in the production of the disease. A repetition of this experiment yielded similar results. It must be mentioned, however, that the strains employed for these experiments were several generations removed from the human body, a fact which deserves consideration in the light of Markl's[73] observations.

This observer found that aside from differences in virulence, a recently isolated strain acted somewhat differently when injected into mice than did the same strain after prolonged artificial cultivation. His observations led him to conclude that the recently isolated strain was mildly infectious, whereas the older strain was incapable of multiplication and exerted a toxic influence only. While we have not undertaken parallel investigations, we have found that certain old laboratory stock cultures were intensely toxic for rabbits, destroying these animals within forty-eight hours, even though only small doses of killed organisms (heated 65° for thirty minutes) were administered. Judging from all the data at hand, we are not warranted in assuming that any very considerable multiplication of the organism occurs in animals inoculated with this coccus.

Mixed infections occurring in connection with experimental inoculations of mice were rather frequently encountered. While in some instances these may have been due to an injury of the gut occurring at the time of injection, in others it was unquestionably a spontaneous infection occurring after inoculation. The purity of the cultures employed was, of course, established before inoculation experiments were undertaken, and the autopsies failed to reveal a perforation of the intestine. These spontaneous secondary infections occurring in the course of animal experiments with the meningococcus are of interest in that they throw light on the work of those who noted a metamorphosis of the meningococcus strains following their passage through animals. The frequent occurrence of these secondary infections following experimental inoculations of meningococci is attributed by von Lingelsheim and Leuchs[71] to a paralytic influence which the meningococcus toxin is supposed to exert on the bactericidal properties of the tissues and of the body fluids. They were unable to obtain any experimental evidence in favor of this view. Our own views regarding this question will be stated in connection with experiments on larger animals bearing on this point.

Inoculation experiments on guinea-pigs. — Our experience with experimental inoculation of guinea-pigs in this connection is somewhat limited, only twenty-five animals having been employed. As we have already indicated, we have found even half-grown guinea-pigs less susceptible to such inoculation than the race of white mice which we used.

The autopsy findings in guinea-pigs closely resembled those in mice and require no special consideration. The bacteriological findings in the peritoneal cavity were variable. In some instances a very considerable number of meningococci were found in the peritoneal exudate, whereas in others prolonged search was required to demonstrate the organism in film preparations. Concerning the blood cultures, we may say that contrary to the findings of Kolle and Wasserman,[46] and in accord with those of Schottmüller[68] and von Lingelsheim and Leuchs,[71] we were usually able to recover the organisms from the heart's blood, though less frequently than in the case of mice. Kolle and Wasserman,[46] in accord with their own observations on guinea-pigs, consider that the infection is purely local, whereas the others are inclined to attribute death to a general invasion not associated, however, with any considerable multiplication of the organisms.

The marked differences observed in the bacteriological content of the peritoneum in different animals receiving intraperitoneal inoculation of the meningococcus, and the conflicting results obtained by different observers and ourselves concerning the bacteriological findings in the blood of such animals suggest that the mode of death induced by such injections is not always the same. Although these differences may be due to and usually are attributed to variations in the powers of resistance exhibited by different animals, it has always seemed to us that while such assumptions are applicable to spontaneous infections of man and animals, they do not apply with equal force to experimentally induced diseases. In the latter the portal of entry is identical, and the dose very much larger than the number of organisms which probably suffice to induce disease spontaneously. Under these conditions, therefore, variations in susceptibility should be less apparent. These considerations lead us to seek factors which might explain the differences in the bacteriologic findings recorded above. Accordingly the following experiments were undertaken. Since these experiments will be made the subject of a separate communication we will omit details and will confine ourselves to a consideration of those findings which are applicable to the question under discussion.

Two rabbits were treated in the following manner:

Each animal was injected, intraperitoneally, with .008 gram of moist meningococcus (twenty-four-hour culture) suspended in normal salt solution. Blood cultures were taken after the lapse of five minutes, thirty minutes, one hour, and eighteen hours. The cultures taken after five minutes, thirty minutes, and one hour had elapsed revealed a pure growth of the meningococcus, whereas the culture taken after eighteen hours had elapsed remained sterile. We repeated these experiments and found that in every instance the organism appeared in the blood immediately after injection, but could not be recovered from the blood after one hour had elapsed. The animals remained well throughout the experiment. A second series of experiments was undertaken to determine whether very slight alterations in the peritoneal cavity would alter these results. Accordingly, two rabbits received intraperitoneal injections of bouillon on each of three successive days. On the day following these injections they were treated in exactly the same manner as the first series. Blood cultures taken at the times specified all remained sterile. The invasion of the blood stream was, therefore, apparently wholly prevented in this series, or reduced to such an extent that they could not be demonstrated in the small amount of blood (one cubic centimeter) which was extracted for culture purposes.

Further experiments along these lines showed that such an invasion of the blood by bacteria introduced into the peritoneal cavity could always be prevented by previous intraperitoneal injections of bouillon. That such injections into the peritoneal cavity of animals conferred upon them a certain degree of immunity has long been known. It has been heretofore attributed, however, to an augmentation of local resistance of the peritoneum to infection. The question arises, therefore, whether in the present instance the failure to appear in the blood of animals thus treated is due to a heightened resistance on the part of the peritoneum, leading to a rapid destruction of the microörganism introduced.

The experiments just cited are, however, incompetent to answer this question. The rabbit is naturally very resistant to experimental meningococcus infection. This organism not only disappears very rapidly from the blood, but is also rapidly destroyed in the peritoneal cavity. This occurs in the untreated as well as in the treated peritoneum. In both sets of animals the peritoneum was found sterile after twelve hours had elapsed. In view of the difficulties surrounding experiments with the meningococcus on this species of animal, we repeated these experiments with organisms which were pathogenic for rabbits and found that we could induce, at will, a generalized infection or a purely local one.

These results lead us to assume that the previous injections had produced a partial clogging of the absorbents of the peritoneum which prevented the invasion of the organisms subsequently injected. This seems more probable than the assumption that the injections produced a degree of local immunity sufficient to contend with the large number of organisms injected, in the short space of time ordinarily required by them to pass from the peritoneal cavity into the blood.

It is conceivable, therefore, that slight alterations in the abdominal lymphatic apparatus of animals, such as might be spontaneously induced by gastro-intestinal disturbances, might be sufficient to alter the course of the disease following intraperitoneal injections; that such alterations may, in part, account for the variations in the bacteriological findings encountered in connection with experimental inoculation of animals with the meningococcus. Our experiments were carried out on rabbits for obvious reasons. We are fully aware that the observations made on rabbits cannot be applied without reserve to the mouse and guinea-pig. It would seem, also, that current views concerning the immunizing power of intraperitoneal injections of bouillon require readjustment.

Inoculation experiments on rabbits. — While these animals, as a rule, do not react acutely to injections of the meningococcus they are, nevertheless, profoundly influenced by such injections. A prompt loss of weight commonly follows such treatment, and in some instances this is progressive, although the injections are discontinued. Some of our immunized rabbits died weeks after the last injection in a very much emaciated condition, and quite a number of animals were lost during the process of immunization. While the exact cause of death could not be established (absence of pronounced lesions and negative bacteriological findings), it seemed probable, judging from the sudden appearance of manifestations of disease in some of the animals, that an intercurrent disease was responsible for their death. In others, however, death seemed to be due solely to the inoculations. As we have already indicated, even small doses of killed or living meningococci occasionally sufficed to cause the death of the animal within twenty-four or forty-eight hours.

The autopsies on animals dying acutely revealed no marked pathological changes, and the bacteriological examinations yielded negative results, even though living cultures were injected. The point of chief interest in connection with the autopsy findings in such animals is the presence of focal hemorrhages in the mucosa of the gastro-intestinal tract. That similar lesions also occur when animals receive sublethal doses of this organism is shown by the presence of small brownish pigmented spots, presumably the remains of previous hemorrhages, in the mucosa of immunized animals dying weeks after receiving the last injection.

The presence of such lesions in man occurring in the course of an attack of epidemic meningitis was first reported by Bettencourt and Franca.[7] This observation was later confirmed by Goeppert[74] and by Radmann.[75] The former regarded these lesions as evidences of a general infection, while the latter suggested the possibility that they may indicate the portal of entry.

In the light of our observations on rabbits, the latter supposition appears untenable.

The occurrence of these lesions in animals inoculated with the meningococcus may also explain the unusual frequency of secondary infections in animals employed for such experiments. It seems probable that such lesions might facilitate the entrance of foreign bacteria. To establish this as a fact we have undertaken a series of experiments which will be considered in a future communication, but may be briefly referred to in this connection.

A number of animals were fed with small doses of typhoid bacilli. While one set was to serve as a control, the other set was immunized in the usual manner by intravenous injections of meningococci. A further control was not fed with typhoid bacilli but was immunized against meningococci. Blood cultures were taken two hours after injecting the killed meningococci.

While we were unable to recover the typhoid bacilli from the blood of animals fed with these organisms and immunized against meningococci, we were able to demonstrate the presence of both group and specific agglutinins for the typhoid bacillus. The control animals, providing they remained well during the period of experimentation, showed no augmentation of the agglutinins for this organism. It must be mentioned, however, that the injection of bouillon is capable of bringing about similar results. While, therefore, the changes produced in the mucosa are not peculiar to meningococcus infections, it seems justifiable to infer from our experiments that this organism, as well as the bouillon, is capable of so altering the mucosa as to permit the passage of foreign organisms present in the intestinal tract.

These observations are of interest in view of the facts recently reported by Symmers and Wilson,[76] who found that sera taken from cases of epidemic meningitis frequently clumped in rather high dilutions an organism which they isolated from tap water. The blood serum from one hundred and thirty-five cases of this disease was examined and only four yielded negative results, while twelve clumped this organism in dilutions of from 1–1,000 to 1–2,000. Sera from one hundred and fifty-five other cases, including healthy adults and patients suffering from various diseases, served as controls. None of these sera agglutinated this organism in dilutions above 1–100.

Very recently Becker and Ruhland[77] reported a case of epidemic cerebro-spinal meningitis in which the blood serum, diluted one hundred times, clumped the typhoid bacillus.

Aside from the macroscopic lesions in the mucosa of the gastro-intestinal tract, which have been observed in connection with epidemic meningitis, we may accept the occurrence of such foreign agglutinins in the blood of meningitis cases as further evidence that the meningococcus or its toxins are prone to involve the mucous membrane of the gastro-intestinal tract.

These experiments further suggest an explanation for the peculiar results obtained by von Lingelsheim[9] with sera taken from rabbits immunized against meningococci. As we have already stated, this observer found that a meningococcus immune serum also agglutinated the Jaeger coccus (Diplococcus crassus) in rather high dilutions. Several explanations for this occurrence have been advanced. Jaeger,[78] intent upon establishing the identity of the two organisms, attributes this reaction to the presence of common agglutinins. Fischer[79] attributes the reaction of the Jaeger organism in the presence of a meningococcus immune serum to the presence of normal agglutinins. This observer found that the Jaeger coccus was unusually sensitive to the action of normal rabbits' serum. Von Lingelsheim,[80] however, while he admits that the organism responds rather readily to normal sera, finds that this hypersensitiveness is lost when the organism is grown on media containing blood. He is, therefore, unwilling to attribute his results to the action of normal agglutinins. Jochman suggests that the animal yielding these results was inadvertently injected with both organisms.

That the agglutinins in a meningococcus immune serum which act on the diplococcus crassus are not related to those concerned in the clumping of the meningococcus may be inferred from the fact that meningococcus immune serum taken from horses agglutinated only the homologous strains. The clumping factor, therefore, appears to be peculiar to rabbits' sera, a fact which in itself seems sufficient to exclude the action of group agglutinins.

Moreover, the absorption tests recorded in a preceding section showed that the substances in a meningococcus immune serum which cause a clumping of the meningococcus cannot be extracted with the diplococcus crassus, and that the converse also holds true. It follows, therefore, that the agglutinins acting on these organisms are not only not identical but are not related to each other, and that the meningococcus is not directly responsible for the presence of agglutinins acting on the diplococcus crassus. The action of group agglutinins may therefore be excluded.

If we accept von Lingelsheim's statement that the organism employed by him did not respond readily to the action of normal sera, the high values obtained with a meningococcus serum cannot be attributed to the action of normal agglutinins. We are forced to conclude, therefore, that the agglutinins acting on the diplococcus crassus appeared in the blood serum in the course of immunization with the meningococcus.

When we consider the fact established in the preceding experiments, viz., that the meningococcus when injected into animals renders the mucous membrane of the gastro-intestinal tract permeable for foreign bacteria, it seems probable that the heterologous agglutinins encountered by von Lingelsheim in animals immunized against this organism owe their presence to bacteria gaining access to the system in the course of immunization.

The bearing which the results of these experiments have on the question of group agglutinins will be considered in a separate communication.

Experiments on larger animals and monkeys have not been undertaken by us. Flexner,[72] von Lingelsheim and Leuchs,[71] and Stuart McDonald[15] succeeded in reproducing a disease in monkeys simulating that occurring in man. This, however, was only possible when the organisms were introduced directly into the spinal canal. The value of these experiments is questionable when we consider that the chief peculiarity of the meningococcus, viz., its peculiar affinity for the leptomeninges, is not taken into account. The ability to produce a meningitis it shares with a great many other pathogenic bacteria, but no other common pathogenic organism shows a similar selective action for the meninges of man. Attempts to reproduce the disease in monkeys by way of the nose (Kolle and Wasserman,[46] Davis[81]) and by intravenous injection of this organism (Davis,[81] McDonald[15]) all failed. Bettencourt and Franca[7] and Kolle and Wasserman[46] obtained negative results with monkeys, although the organisms were introduced into the subdural space.

Councilman, Mallory and Wright[5] succeeded in producing meningitis in one goat by intraspinal injections of the genuine meningococcus. Others working with genuine meningococcus strains obtained negative results (Albrecht and Ghon,[6] Bettencourt and Franca,[7] von Lingelsheim and Leuchs,[71] and Stuart McDonald [16]). Still others who recorded successful infections of the leptomeninges of goats were dealing with organisms, the meningococcic nature of which is open to question (Heubner,[82] Weyl[88]). In view of these negative results it cannot be said that the goat is especially susceptible to experimental infection with this organism.

GENERAL SUMMARY AND CONCLUSIONS CONCERNING THE AGGLUTINATION REACTIONS WITH HUMAN SERA, THE BACTERIOLOGICAL DIAGNOSIS OF EPIDEMIC CEREBRO-SPINAL MENINGITIS, THE VIABILITY OF THE MENINGOCOCCUS, AND ITS PATHOGENICITY FOR ANIMALS.

Agglutination reactions with human sera.—Sera derived from cases of epidemic cerebro-spinal meningitis may contain specific agglutinins. In testing for the presence of such agglutinins it is essential to carry out parallel tests with a normal serum. Certain meningococcus strains are unusually sensitive to the action of non-specific agglutinins.

Bacteriological diagnosis of epidemic cerebro-spinal meningitis. — 1. In two hundred and ten cases of this disease a bacteriological examination of the spinal fluid withdrawn during life served to establish a positive diagnosis in one hundred and ninety-four instances (92.4 per cent). A microscopic examination of this fluid furnished positive results in 82.46 per cent of the cases. Cultures yielded positive results in 85.88 per cent of the cases. For the successful isolation of the meningococcus from the lumbar puncture fluid, it is important to prepare the cultures shortly after the withdrawal of the fluid and to place them under suitable conditions for growth immediately.

2. Gram negative cocci, which were not identical with the meningococcus, have only rarely been encountered in lumbar puncture fluids. For practical purposes the detection in films made from such fluids of a Gram negative coccus, having the morphological and staining characteristics of the

meningococcus, suffices to establish a diagnosis of this disease providing the examination is made shortly after the withdrawal of the fluid, and the cellular elements present indicate the existence of an acute meningitis. An intracellular position of the organism enforces the diagnosis.

3. For the positive identification of Gram negative cocci recovered from the naso-pharynx or parts more or less removed from the central nervous system, it is necessary to expose the organism to all the tests considered in the preceding sections.

4. By the use of the combined cultural and microscopic method, a positive diagnosis was established in thirty out of thirty-three cases examined at autopsy. In one of the negative cases agglutination tests served this purpose.

5. Instances of mixed infections in this disease are rare. The alleged occurrence of the meningococcus in the spinal fluid withdrawn from cases of tuberculous meningitis requires confirmation.

Viability of the meningococcus. — It is now generally admitted that the meningococcus is unusually sensitive to external influences. Our investigations lead us to endorse this view. While twenty-four-hour growths do not survive desiccation for more than twenty-four hours, certain of the strains which were allowed to grow for forty-eight hours resisted drying for seventy-two hours. In culture media protected against drying and exposed to incubator temperature, several strains remained alive three hundred and thirty-three days without transplantation.

Pathogenicity for animals. — 1. The meningococcus is only moderately pathogenic for the usual laboratory animals. Large doses of this organism injected into the peritoneal cavity of mice and half-grown guinea-pigs are usually attended by a fatal issue. Rabbits are more resistant.

2. Judging from the data in hand, no considerable multiplication of the meningococcus occurs within the animal

body. The injection of organisms killed by drying also causes the death of these animals. The dose required is twice that necessary to attain this end with the living organism.

3. We have noted marked differences in the bacterial content of the peritoneum in animals dying as the result of intraperitoneal injections with the meningococcus. In mice dying of experimental inoculations the meningococcus could usually be recovered from the blood. In guinea-pigs positive blood cultures were obtained less frequently. These differences in the bacteriological findings we have attributed in part to local conditions existing in the peritoneum of animals before the microörganisms were administered.

This view is based on the fact that in normal rabbits the meningococcus passes into the circulating blood immediately after it has been injected into the peritoneal cavity, while previous intraperitoneal injections of bouillon suffice to confine the organism, subsequently administered, to the peritoneal cavity. Purely local conditions may, therefore, suffice to alter the distribution of the organisms introduced into the peritoneal cavity.

4. Mixed infections in animals receiving injections of the meningococcus are common.

5. In rabbits we have shown that injections of the meningococcus render the mucosa of the intestinal tract more permeable for the typhoid bacillus (or its products) administered with the food. Animals thus treated developed agglutinins for the organisms administered by mouth, while no augmentation of the agglutinins was observed in the control animals which were fed with typhoid bacilli but received no injections.

These experiments suggest an explanation for the frequent occurrence of mixed infections in animals receiving experimental inoculations of the meningococcus, and also offer an explanation for the appearance of heterologous agglutinins in the sera of such animals and in the sera of individuals suffering from epidemic cerebro-spinal meningitis.

MODE OF DISSEMINATION OF THE MENINGOCOCCUS.

Concerning the mode of transmission of epidemic cerebrospinal meningitis, most observers are agreed that it takes place directly from individual to individual, and that its cause gains access to the body by way of the respiratory passages.

The studies carried out in connection with the extensive epidemics which prevailed in this country and abroad during the winter of 1905, have furnished abundant evidence in favor of this view. The almost constant presence of the causative agent in the throats of individuals suffering from this disease (93.8 per cent von Lingelsheim[9]) and the frequent occurrence of so-called meningococcus carriers in the immediate surroundings of such individuals, renders such a conception extremely plausible. The most weighty argument, however, in favor of a direct transmission of the disease is to be found in peculiarities of the meningococcus itself. The fact that the organism is extremely sensitive to external influences renders any other mode of transmission improbable. In this connection, however, it must be borne in mind that the conditions under which such hypersensitiveness on the part of the meningococcus is determined experimentally are not comparable with those found in nature. In fact it seems probable that the degree of resistance to drying is not quite as limited as our own experiments seem to indicate. In reviewing the table embodying the records of these experiments, it will be found that while the majority of strains were apparently destroyed after twenty-four hours' desiccation when plain broth was employed for the recovery of the organism, several strains resisted drying for seventy-two hours when ascitic broth was provided for their subsequent development. It is fair to assume, therefore, that the results would have been even more favorable if a more fertile medium could have been selected for the recovery of the dried organism. When we consider that the meningococcus thrives and is able to maintain its existence in the throats of certain individuals in spite of all efforts to eradicate it, it must be admitted that such throats offer more favorable conditions for the meningococcus than the culture media usually provided for them. These considerations open the door to the possibility of indirect infection, and although such a possibility might appear to be somewhat remote, sufficient can be said in its favor to justify a continuance of the precautionary measures heretofore adopted to eradicate this possible source of infection. The longevity of the meningococcus in the moist condition also deserves to be mentioned in this connection. It remains to be stated that transmission of the disease to distant parts through the agency of lifeless objects has not been proved.

While direct transmission from person to person may be accepted as the usual mode of propagation, it is a matter of considerable interest and importance that hospital patients suffering from the epidemic form of meningitis rarely transmit the disease to those who attend them or to patients occupying the same ward. This may be attributed in part to the natural immunity enjoyed by most individuals, to the improved hygienic conditions surrounding such patients, to the measures adopted by the attendants to prevent such infection and, finally, to the somnolent or apathetic condition of the majority of patients when they first enter the hospital. Moreover, the period during which the danger of transmitting the disease exists is limited by reason of the rather rapid disappearance of the meningococcus in the throats of individuals actually suffering from this disease. According to Flügge's[84] investigations, the meningococcus usually disappears from the nasopharynx of meningitis cases after the fifth day. Attempts made by us to isolate the organism from the nasopharynx after the second week usually failed, which is in accord with the experiences of others.

Instances of hospital infection have rarely been recorded. In reviewing the records of the New York Hospital, however, we find that three nurses have acquired this disease while attending cases of meningitis. In every instance the patients in question were adults, who cannot be controlled as readily as are children and are more given to sputtering and projecting saliva than are young children suffering from a serious illness. In support of the comparative innocuousness of young children suffering from meningitis, the following observations may be mentioned. The children's ward at the New York Hospital is rarely wholly free from such cases during the winter and spring months and the number present at one time not infrequently rises to six or seven. This ward usually holds from eighteen to twenty patients. Repeated bacteriological examinations of the nasopharynges of nurses and attending physicians, and of all the inmates of this ward suffering from diseases other than meningitis, failed to reveal the presence of the meningococcus. While similar examinations of adults occupying the wards harboring meningitis cases also yielded negative results, the number of meningitis cases in such wards being relatively small, these results are less significant than are those obtained in the children's department.

Whether the same applies to patients and attendants confined to close quarters, such as our New York tenements provide, seems unlikely, when we consider that the meningococcus has been repeatedly demonstrated in the throats of close relatives of cases of meningitis. However, this fact alone offers no solution of the problem, since a simultaneous infection of several members of a family from the same source cannot be excluded.

Bacteriological examinations of the throats of hospital authorities whose business it is to interview the relatives of patients yielded negative results.

The second, and probably the most important, contribution made to this subject in recent years is a knowledge of the fact that the meningococcus is very frequently present in the nasopharynges of apparently healthy individuals who have come in more or less close contact with cases of epidemic meningitis. These individuals, as a rule, develop none of the manifestations of this disease and the lesions in the pharynx produced by the meningococcus are usually so insignificant as to escape notice.

The occurrence of the meningococcus in the throats of healthy individuals was first demonstrated by Albrecht and Ghon[6] in 1901, and this observation has since been confirmed by a large number of investigators. Among these we may mention Lord,[86] Kiefer,[87] Collins,[88] Weichselbaum and Ghon,[89] Meyer,[90] von Lingelsheim,[9] Flügge,[54] Kolle and Wassermann,[46] Kutscher,[70] Ostermann,[91] and Hasslauer.[92]

These later investigations have developed the fact that the number of meningococcus carriers is relatively very great. According to Flügge's[84] estimate, they are from ten to twenty times more numerous than are the cases of meningitis developing during the same period. According to the same observer, seventy per cent of the individuals living in the immediate neighborhood of meningitis cases act as meningococcus carriers. As showing the frequency of meningococcus carriers among members of families in which one member has developed the disease, the work of Ostermann[91] may here be mentioned. This observer found that no less than seventeen out of twenty-four members of such families acted as carriers, while the examination of ten individuals who, though similarly exposed to infection but not inhabiting the same dwellings, yielded negative results. Similar observations were made by Huber,[85] Bochalli,[107] and others. These figures also serve to show how very great is the percentage of individuals who are immune to this disease. This is further supported by the fact that, as a rule, only one member of a family acquires the disease. Instances of a more generalized predisposition have, however, been recorded.

Until very recently meningococcus carriers had only been found during an epidemic and only among individuals who had come in more or less close contact with cases of this disease. Kutscher,[70] however, has in four instances demonstrated their occurrence apart from epidemics. These were observed in Berlin during a time when an epidemic of this disease did not prevail. Moreover, a direct connection with sporadic cases could not be demonstrated. While these observations serve to establish the fact that meningococcus carriers do occur apart from epidemics, the large number of negative results (Kolle and Wasserman,[46] one hundred and twelve; von Lingelsheim,[9] one hundred and twenty-five; Kutscher,[70] fifty-two) recorded in connection with throat examinations carried out on individuals during a time when no epidemic prevailed or among individuals who had not been exposed to infection, indicate that such occurrences are rare. These isolated cases, however, are important, since they furnish a clue as to the manner in which this disease is perpetuated. Weichselbaum[58] suggests the possibility that the meningococcus once established in the nasopharynx of certain individuals may remain there permanently. Flügge[46] expresses similar views.

The occurrence of adult meningococcus carriers explains many of the peculiar features of an epidemic, viz., the fact that the epidemic begins gradually, differing in this respect from an epidemic of typhoid and cholera, that it does not confine itself to any circumscribed district, that it is very apt to appear almost simultaneously in localities far removed from each other and, finally, the fact that it tends to spread along the main channels of traffic. When we consider all the facts bearing on this phase of the question, namely, the comparative innocuousness of patients suffering from the disease, the short period of incubation, the relatively small number of meningitis cases compared to the number of individuals who harbor the causative agent and, furthermore, the fact that only a limited number of individuals are exposed to infection from actual contact with meningitis cases, it is evident that the chief part in the diffusion of this disease is played by healthy meningococcus carriers. These facts further explain why preventive measures directed toward the patient and his immediate environment have not been effective in checking the spread of the disease. They do not explain, however, why in one instance the disease appears sporadically and in others epidemically. It seems probable that meteorological conditions may be important factors in determining these differences. The recent epidemic in Germany prevailed during a time when there was a conspicuous absence of sunny days; their number falling far below the normal for the locality involved. Aside from this observation, however, we have no data which might serve to explain this peculiarity of the disease. The assumption that its cause is to be found in an augmentation of the virulence of the meningococcus may be advanced, but the fact that such augmentation does not occur in the passage of the organism through susceptible animals deprives this supposition of much of its value.

Portal of entry. — That the meningococcus gains access to the system by way of the respiratory passages is now generally accepted. The frequent and early appearance of the meningococcus in the nasopharynx of individuals suffering from this disease, combined with the fact that this organism is not a common inhabitant of the human throat, excepting during a period when an epidemic of meningitis prevails, leaves little room for doubt as to the correctness of this view. Opinions to the contrary have already been referred to and their validity questioned in a previous section. There remains, however, another possible source of the specific organisms found in the throats of meningitis patients. In one instance we demonstrated during an exacerbation of the disease a large number of meningococci in the throat of a patient in which repeated previous examinations were negative. This observation suggested the possibility that the bacteria had escaped from the brain by way of the lymph channels at the base of the skull. The organisms in this instance appeared in the throat on the seventy-sixth day of the disease at a time when, according to all previous experiences, the throat should have been free from meningococci. Our experiences with children occupying the same ward with meningitis cases do not support the supposition of a reinfection from outside sources, although it must be admitted that visiting relatives might have served this purpose in the present instance.

Experiments to determine whether the meningococcus is excreted by way of the nasopharynx may here be recorded. For this purpose we selected young puppies which, according to a verbal communication of Dr. C. W. Field of the

New York Health Board, are susceptible to intracranial infections with the meningococcus.

Accordingly, two puppies were trephined, well forward, so as to make the cribriform plate accessible. One animal received a subdural injection of one-half cubic centimeter of a dense suspension of meningococci. The second animal received an intranasal injection of two and one-half cubic centimeters of a similar emulsion by means of a catheter passed well up into the nose. It was believed that the injury to the nasal mucosa attending this operation was sufficient to allow the passage of bacteria if they selected this path to enter the system. Cultures on ascitic agar taken after the lapse of two, four, six, and twenty-four hours from the nose in the first instance and from the base of the brain in the second, failed to reveal meningococci in either locality. Cultures from the base of the brain remained sterile with the exception of one plate (six hours), which showed a few staphylococcus aureus colonies. Films made from the nose of the animal receiving the subdural injection showed a few Gram negative cocci. Similar organisms were, however, found in films made from the nose of the animal before the experiment was undertaken. These organisms failed to grow on cultures taken before and during the experiment.

This experiment would indicate that the organism does not pass through the base of the skull in either direction, but the fact that both animals remained perfectly well deprives this experiment of any real value. Flexner,[78] experimenting with a race of monkeys which is susceptible to intraspinal injections with this organism, demonstrated the presence of Gram negative cocci in the nasopharynges of animals having previously received an intraspinal injection of meningococci. So far as we are able to learn, however, these organisms were not identified culturally. In view of the occurrence of Gram negative cocci in the nose and throat of most of the animals examined by us, we cannot accept this observation as establishing the point under consideration.

Moreover, we wish to add that even if such an occurrence could be positively established, it does not affect the validity of the arguments adduced in favor of the contention that the nasopharynx (respiratory tract) is the portal of entry for the meningococcus. Von Lingelsheim[9] and E. Meyer[90] were able to demonstrate the existence of a meningococcic pharyngitis prior to the appearance of symptoms of meningitis.

Paths selected by the meningococcus to reach the meninges. — While complete unanimity of opinion may be said to prevail concerning the portal of entry, the question as to the paths selected by the meningococcus in reaching the brain is still under discussion. Of the two possibilities, viz., direct extension of the process from the nasopharynx through the base of the skull to the brain and primary invasion of the blood stream with subsequent localization in the meninges, the earlier observers were inclined to accept the former.

The evidence, however, which can at present be adduced in favor of this view is not very considerable. While the close proximity of the roof of the nose and mouth to the base of the brain is very suggestive, it is of no value as proof of a direct extension. The fact that the exudate is usually most abundant at the base of the brain is also capable of another interpretation; in fact, can be explained on purely mechanical grounds. The early involvement of the structures surrounding the hypophyseal gland advanced by Westenhoeffer[94] in support of the doctrine of direct extension is not peculiar to this form of meningitis. It occurs in connection with tuberculous meningitis in which the bacillus unquestionably reaches the brain by way of the blood stream. In fact, Westenhoeffer,[95, 96] himself one of the most active exponents of the theory of direct invasion, has done most towards making this supposition untenable. He has shown that the perihypophyseal suppuration is confined to the superior and lateral surfaces of this organ, and pointed out that if a direct invasion through the body of the sphenoid had occurred, the inferior surface should show the most prominent lesions. In two instances we have made cross-sections of the hypophysis and sphenoid bone, but have failed to find any evidence in favor of a direct extension through this portion of the base of the skull.

Westenhoeffer has further established the fact that the lymphatics at the base of the skull, and more particularly those surrounding the carotid vessels, show no evidences of invasion along these channels. A microscopic examination of the nerves emerging from the base of the skull also failed to establish invasion along these paths. On the contrary, he found that an involvement of these structures occurred by an extension of the process from within outward. Lastly, he found the same to hold true for the spinal nerves.

To sum up, therefore, there is no evidence in favor of a direct invasion of the meninges, and in the absence of such evidence the facts thus far recorded may be advanced as negative evidence in support of another mode of entry for these organisms.

Concerning the second possibility, viz., a primary invasion of the blood stream with a secondary involvement of the meninges, many facts have been advanced in support of such a view. The early appearance of the meningococcus in the blood in a considerable number of cases, the appearance of general sepsis suggested by some patients early in the disease, the lesions in parts far removed from the central nervous system found at the autopsy of individuals who succumbed to the disease within twenty-four hours of its inception, and finally the appearance of characteristic lesions in the eye synchronously with manifestations referable to the central nervous system, all point toward an early generalization of the meningococci but are not competent to prove that such a general infection actually antedated the meningeal involvement.

That such a generalization of the infecting organisms may precede its lodgment in the meninges appears to be established by the cases of Solomon,[97] of Martini and Rhode,[98] of Marcovitch,[99] and of Andrewes.[33] These observers isolated the meningococcus from the blood before symptoms referable to the meninges were at all pronounced. The question arises, however, whether such isolated occurrences furnish valid data upon which to base the contention that a primary infection of the blood with a secondary localization in the meninges is the usual course of events in this disease. Aside from the fact that the number of such cases thus far observed is too small to warrant such a generalization, attention must be called to the fact that the clinical course of the disease in these cases differed from that usually observed. The investigators referred to are all agreed that the septic process completely dominated the clinical picture. We may infer, therefore, that an actual septicemia existed, yet from the bacteriological point of view such an occurrence in epidemic meningitis is rather rare. The number of negative blood cultures far exceeds the number of positive findings, which militates against the assumption that blood infection frequently prevails during the course of this disease.

In a series of forty-one cases we succeeded in isolating the meningococcus from the blood in eleven instances, and while we contended at the time that improvement in the technic and the use of more suitable culture media would yield a greater number of positive results, we hesitated to regard these findings as evidences of a septicemia for the following reasons:

The number of organisms found in the blood, although not definitely established, appeared to be small, since with one exception, only one, or at the most two, of three culture flasks usually employed yielded positive results. The amount of blood employed was five cubic centimeters distributed among three culture flasks. Moreover, in four cases in which positive results had been obtained, blood cultures were repeated, but the organism was recovered a second time in only one of these. These results suggest that a transient invasion of the blood stream existed in these cases, while in the cases referred to above an actual septicemic process appeared to exist.

We are not satisfied, therefore, that the blood findings thus far recorded furnish conclusive evidence in favor of a hematogenic mode of origin. If we turn to the clinical manifestations of this disease we find one feature which strongly suggests that the organism first gains access to the blood and secondarily localizes in the meninges.

In the vast majority of instances the epidemic form of meningitis begins with alarming suddenness, and the clinical picture, as a rule, reaches its full development within a very short time. In considering these facts the question arises whether such a prompt and complete development of the disease is compatible with the assumption that the organisms gain access to the meninges at some particular point in the skull. If we turn to other forms of meningitis in which such a direct infection of the meninges of the brain is reasonably certain, for example in otogenic or traumatic forms of meningitis, we find that, as a rule, the onset is gradual and the development of symptoms irregular, suggesting in the beginning a more or less limited involvement of the meninges. These differences in the mode of development of the two types of meningitis we are not disposed to attribute to any peculiarities of the organisms concerned in their production, nor to any peculiar condition which may prevail in the meninges in the one instance and be absent in the other, but to the manner in which the organisms gain access to the structures involved.

There are no good reasons for assuming that the meningococcus if it gained access to the brain through an opening in the skull would act otherwise than a streptococcus or pneumococcus entering by the same channels.

Assuming that an organism reaches the meninges by direct channels, excessive virulence or a total lack of resistance on the part of these structures would alone explain the prompt and complete development of the symptoms.

Neither of these prerequisites can be rationally assumed to exist. The fact that many patients recover precludes the idea that lack of resistance on the part of the individual accounts for the sudden development of the disease and the favorable prognosis in this type of disease compared with forms of meningitis due to other bacteria, renders the supposition of excessive virulence on the part of the meningococcus untenable.

These considerations, we believe, also tend to show that the doctrine of direct invasion of the brain by the meningococcus is, on the whole, untenable.

That exceptions to the rule occur may be admitted, but even in instances in which the disease develops gradually, a direct invasion need not be assumed. Unusual resistance on the part of the individual or reduced virulence on the part of the infecting organism would account for such an occurrence. In the following section we shall show that the mode of onset usually observed in this disease also occurs in animals receiving intravenous injections of an organism which finally lodges in the brain and produces the clinical symptoms and anatomical findings of meningitis.

EXPERIMENTAL MENINGITIS.

All attempts to produce meningitis in animals by inoculation of the meningococcus in parts more or less removed from the central nervous system have failed. In monkeys, under these conditions, injections of the meningococcus have yielded negative results, although these animals are susceptible to direct infections of the meninges with these organisms.

In view, therefore, of the total absence of experimental proof of a hematogenous origin of meningitis, we may record, briefly, experiments which establish this possibility, although undertaken with an organism not related to the meningococcus. For our present purpose it will be necessary to record only the most important facts concerning these experiments. A full report will be published with Dr. I. Strauss, who assisted us in this work. The organism employed for these experiments is an encapsulated chain-forming coccus closely resembling the streptococcus mucosus, morphologically and culturally. It was isolated from the lung at autopsy in a case of lobar pneumonia which presented symptoms of meningismus during life. Two bacteriological examinations of the spinal fluid during life yielded negative results. Unfortunately, permission for a post-mortem examination of the brain was refused.

Injected into the circulating blood of a rabbit, this organism produced, aside from loss of weight and slight irregularities in the temperature curve, no manifestations of disease until three days had elapsed. On the fourth day, symptoms referable to the central nervous system developed. There was marked retraction of the head, which could not be made to assume its normal position without apparently causing great pain. The animal shrieked repeatedly and was subject to general convulsions occurring at short intervals. During the quiescent periods the animal did not appear to be seriously ill and, it is important to add, presented none of the appearances of an animal suffering from general septicemia. In this instance the disease extended over a period of four hours only and terminated in death. A second rabbit received an intravenous injection of a mixture of this organism and the meningococcus, a third rabbit was injected with a pure culture of this organism in the region of the tonsil. These animals died during the night of the fourth and fifth days respectively, although the day before death such an issue was not expected. We assume that the clinical manifestations of cerebro-spinal involvement were also present in these cases just before death, since the anatomical findings at autopsy were identical with those noted in the first animal. A fourth rabbit, injected intravenously at the same time but receiving twice the dose injected into the peritonsillar region of the third rabbit, recovered without developing symptoms of meningitis. Aside from loss of weight and slight irregularities in the temperature curve, this animal was apparently not influenced by the injection. These experiments were repeated with other rabbits, and although we took the precaution to employ for this purpose organisms isolated from animals dying as a result of similar injections, we were unable to reproduce the disease. We may infer, therefore, that passage through the rabbit did not augment the virulence of this organism for the same species. Attempts to reproduce the disease with cultures which had not been passed through animals also failed.

Autopsy and bacteriological findings.—The essential lesions were confined to the central nervous system and kidneys. The latter organs in the first animal showed no pronounced macroscopic changes, although a microscopic examination revealed the lesions of an acute exudative nephritis. In the two remaining animals numerous hemorrhages into the substance of the kidneys were found. A macroscopic examination of the brain and cord showed no prominent lesions. The cerebral convolutions showed some flattening and the pial vessels were moderately injected. At the base of the brain there was a moderate amount of turbid fluid, which upon microscopic examination revealed numerous pus cells and innumerable cocci, corresponding morphologically and in their staining reactions to those used for the experiments. Films made from the cortex, on the other hand, showed only a few leucocytes and only a small number of encapsulated cocci. A microscopic examination of the blood showed no organisms in two instances and only a few in the third. Cultural examinations showed the presence of the organisms in the blood of all three rabbits, but the number of colonies which developed on plates was small compared to the number found on plates made from the meningeal exudate.

Cultures from the spleen and kidneys also showed a growth of the same organism, while cultures from the pleura, peritoneum, and pericardium remained sterile. The meningococcus, which was simultaneously introduced into one of the animals, could not be recovered in cultures or demonstrated in films made from the brain or blood.

Histological examination of the brain and cord revealed the existence of a purulent cerebro-spinal meningitis and, what is important for our present purpose, showed a distribution of the lesions identical with that found in the epidemic form of meningitis in man.

A microscopic examination of the kidneys revealed the lesions of an acute exudative nephritis, associated in two instances with hemorrhages. While the changes in the kidneys removed from the first and third animal were pronounced, they were less marked in those removed from the second.

Judging from the manner of death observed in one of the animals and from the anatomical findings in all, we are inclined to attribute the immediate cause of death to the lesions in the central nervous system and to regard the changes in the kidneys as contributing factors.

It may be urged that the bacteriological findings detailed above indicate the existence of a septicemia; but when we consider the large number of organisms introduced, the occurrence of an actual multiplication in the circulating blood, which constitutes a septicemia, does not seem probable.

The conclusions which may be drawn from these experiments are:

1st. That the organisms in question have a special predilection for the meninges.

2d. That the peculiar distribution of the lesions, viz., the special involvement of the base of the brain, is not due to the manner in which the organisms gain access to the brain, but to other factors. Among these we may mention gravity and the roominess of the sub-arachnoid spaces in this locality.

3d. That the meninges of the brain and cord are simultaneously involved. Owing to the horizontal position usually adopted by these animals, a prominent part cannot be assigned to the influence of gravity in causing an extension from the brain to the cord.

Moreover, if we assume that the organism in question has a special predilection for the meninges, there is no reason to suppose that the meninges of the cord are more, or less, resistant than those of the brain.

While we believe that the second and third propositions require no further substantiation, the validity of the first may be questioned, since an uncomplicated cerebro-spinal meningitis was not induced in any of the animals. The coexistence of an acute exudative nephritis in three animals raises the question whether the kidney lesions were indirectly concerned in determining the localization in the meninges of the injected organisms. This possibility must be admitted in view of the fact that changes in these membranes are not infrequently associated with renal affections in man, and because of the probability that similar changes may also occur in the course of renal diseases in animals. It is possible, therefore, that alterations in these membranes brought about by the renal affections prepared the ground for the reception of the bacteria. That the injected bacteria do not lodge immediately in the leptomeninges is indicated by the time which elapses before any symptoms arise, referable to the central nervous system, and by the fact that the changes found at autopsy in these membranes are of very recent date. It may be accepted, therefore, that some preparation of the membranes is necessary before the organisms can gain a foothold. The question which concerns us is whether these changes are of a general character brought about by preexisting disease of the brain or induced as a result of disturbances arising in the course of diseases of other organs, or whether they are more or less specific in character, *i.e.*, brought about by the toxic substance elaborated by the injected bacteria during life or liberated after their death. In our animals we are inclined to accept the latter view.

[Since writing this paper experiments bearing on this point have been undertaken. Rabbits were injected subcutaneously with small doses of uranium nitrate or arsenious acid.

These injections were repeated until the urine showed distinct evidences of nephritis. Following this treatment the animals received intravenous injections of a virulent pneumococcus culture. In four animals thus treated none developed symptoms referable to the meninges and an examination of the brains after death failed to reveal evidences of meningitis.

One of the control animals receiving pneumococci only developed nephritis and died The examination of the brain in this instance also failed to show evidences of meningitis.

It seems probable, therefore, that the kidney lesions were not concerned in determining the localization of the encapsulated streptococcus.]

The changes associated with marked lesions in the kidneys in man are not confined to the leptomeninges. Other serous membranes are not infrequently involved. If we assume, therefore, that no special relationship existed between the leptomeninges and the bacteria in question, it would be difficult to explain the sole and predominant involvement of that membrane. The pleura, pericardium, and peritoneum in these animals were normal throughout and the cavities free from bacteria. The involvement of the kidneys may be explained on the ground that they were engaged in excreting the noxious products introduced into the circulation. The use of smaller doses of this organism might have sufficed to produce meningitis in these animals without at the same time causing marked changes in the kidneys.

We regret that a deeper insight into these problems could not be attained, owing to the rapid reduction of the virulence and the early death of the bacteria employed for these experiments.

In reviewing the records detailed above it is evident that the disease induced experimentally in rabbits closely resembles that occurring spontaneously in man and, although the causal agents differ, it seems justifiable, in the absence of other experimental data, to draw certain conclusions from this similarity.

As we have already stated, the animals showed no pronounced manifestations of disease during the first three or four days following the injections; in fact, daily inspection of the animals during this time led us to believe that the injections would be without effect. After this period of incubation, the disease set in with striking suddenness, the clinical picture presented was clearly one of meningitis, and the disease ran a rapid course. In our animals the duration of the disease did not exceed eight hours. The same description might be applied to fulminating meningitis in man and, even in those cases which are destined to recover, the most alarming symptoms occur during the earliest periods of the disease. Prodromal symptoms in man are also usually absent or very slight. Speaking generally, the pathological findings in the central nervous system of these animals and man are also identical.

Reasoning by analogy, therefore, a hematogenous origin of epidemic cerebro-spinal meningitis becomes probable.

As an argument against such an origin it may be urged that the meningococcus is only rarely encountered in the circulating blood. While it must be admitted that the number of negative findings greatly exceeds the positive results, an analysis of the animal experiments cited above appears to offer an explanation for this fact. The number of organisms injected into the blood was very large (one slant agar culture), yet the number found in the blood at autopsy, four hours after the inception of the disease, was small.

We may infer from this that an actual multiplication of the organisms in the blood did not occur, and that on the contrary a rapid destruction had taken place. Moreover, it is quite possible that the number would have suffered further reduction if the animals had survived for a longer period. The point, however, which we wish to emphasize is the fact that the number of organisms present in the blood, before symptoms of meningitis developed and therefore before any indication for a blood culture had arisen, was far in excess of the number found at autopsy. Whether or not the disappearance of the bacteria from the circulation is solely due to the action of the blood is difficult to answer. Apart from the possibility that many of them may be discharged from the body by various means (lesions in the kidneys), it is theoretically conceivable that the inflamed structures may act as a filter, so that many of the organisms may be removed from the blood mechanically. It is not improbable, therefore, that the meningococcus may be present in the blood of man before symptoms of meningitis arise and subsequently diminish in number or disappear altogether.

The large number of negative results obtained after the full development of the disease may thus be accounted for.

THE ETIOLOGICAL RELATIONSHIP OF THE MENINGOCOCCUS, PNEUMOCOCCUS, AND STREPTOCOCCUS MUCOSUS TO EPIDEMICS OF MENINGITIS.

The question as to the causal relationship of these organisms to epidemics of meningitis has been variously answered by different observers. While many seem inclined to accept the meningococcus as the sole cause of epidemics of meningitis, others, among them such authorities as Weichselbaum[16] and Netter,[104] contend that the pneumococcus may also act in this capacity. On the other hand, Schottmüller[68] asserts that the causal relationship of the pneumococcus to epidemics of meningitis has not been established. A review of the literature leads this observer to conclude that the recorded instances of epidemics of this disease which were not due to the meningococcus were brought about by the streptococcus mucosus. The three species just mentioned are, so far as we are aware, the only organisms which have been seriously considered in connection with epidemics of meningitis.

If we propose to investigate the validity of these various contentions, two methods are at our disposal. We may turn to the literature and attempt to identify the organisms described and reported as the causative factors in different epidemics, and base our conclusion on such an analysis, or we may endeavor to arrive at the truth by a consideration of the pathogenic properties of the organisms reputed to be concerned in the production of such epidemics. The former method has yielded very little reliable information, although it alone has been followed, so far as we are aware, by all observers who have contributed to this phase of the subject. All that we have been able to establish positively from a review of the literature is the fact that certain epidemics of meningitis were not due to the meningococcus. Regarding the nature of the organisms concerned in the production of these epidemics, it seems venturesome to us to declare their identity with one or other of the organisms known to be the causative factors of primary meningitis. The descriptions given are either incomplete or they do not harmonize in their totality with what is known of the pneumococcus on the one hand, or the streptococcus mucosus on the other. For example, the organism encountered by Bonomè[102] in a small epidemic occurring in Italy is regarded by Weichselbaum as a pneumococcus, by Schottmüller as the streptococcus mucosus, and by Bonomè as an organism quite distinct from the pneumococcus. While we are aware that a very close relationship between the pneumococcus and the streptococcus mucosus is assumed to exist by many observers, we here draw a sharp distinction between these organisms for reasons that will be apparent presently.

In view of the uncertainties surrounding the recognition of the organisms isolated by different observers, we believe that an analysis of the pathogenic properties of the species believed to be concerned in the production of epidemics of meningitis will furnish us with more reliable data. The views which form the basis for such an analysis may be embodied in the following propositions:

1st. An organism capable of producing a definite disease in an organ or tissue with any degree of regularity does so by virtue of a special predilection, or, we may say, affinity, which it has for these structures. This, we believe, holds true, providing the structures are not exposed to direct infection and are not concerned in the excretion of the noxious products elaborated by such organisms. To avoid misunderstanding, we may say that the term affinity is not used in the sense that the bacterium itself favors a localization in a given structure. As we have already indicated, it is more than likely that its products, either those elaborated by it during life or liberated after its death, prepare the ground for such a localization. In other words, the structures thus involved are relatively hypersensitive to the particular poison elaborated by a given microörganism.

2d. An organism showing no such predilection for a given structure is not apt to reproduce a disease characterized by the sole or predominant involvement of this structure. When it does so, the prevalence of unusual conditions must be assumed.

3d. If it can be established that a bacterium has no such relationship to a given structure, it can be excluded from consideration as a factor in epidemics; while if such a relationship can be established, the possibility that such an organism is capable of producing an epidemic must be admitted, even though it has not been encountered in this connection.

That the meningococcus exhibits a special predilection for the leptomeninges of man seems extremely probable. The fact that its chief point of attack when it gains access to the internal structures of the body is almost invariably the central nervous system, supports this view. All the facts adduced in favor of a hematogenous origin of meningitis may also be arraigned in its support.

While, therefore, abundant evidence is at hand to enforce the contention that the meningococcus displays a special affinity for the meninges, it must be admitted that positive proof of this cannot be advanced. The close proximity of meninges to the usual portal of entry for the meningococcus opens the door to the possibility of a direct infection of these membranes, in which case it would be unnecessary to assume a selective action on the part of this organism to explain the constant and predominant involvement of the meninges.

But, as we have already stated, evidence in favor of such a mode of infection is wholly wanting, and if assumed it would be difficult to explain why other pathogenic organisms which frequently lodge in the nasopharynx are not equally prone to infect the meninges. Moreover, there are instances on record in which the organism apparently entered by way of the lungs.

The early involvement of the lungs in the cases of Goeppert[74] and of Jacobitz[49] may be thus interpreted.

Finally, we may mention the fact that the only other organism whose causal connection with epidemics of meningitis has been established, as we shall show directly, actually exhibits a special predilection for the leptomeninges of rabbits. The fact that one organism is known which selects the meninges in preference to other structures, adds to the probability of the assumption that such a relationship exists between the meningococcus and these membranes.

The diplococcus pneumoniæ, on the other hand, exhibits no such relationship to the meninges. In support of this and in support of the contention that this organism is incapable of producing an epidemic of meningitis, the following arguments may be advanced:

When we consider the frequency of pneumococcic infections in man and the rarity of meningitis induced by this organism, such a relationship seems improbable; and when we further consider that in almost every case of lobar pneumonia the organism gains access to the blood, yet fails to lodge in the meninges in the vast majority of instances, the assumption that it has no special affinity for the meninges amounts almost to a certainty. The fact that a pneumococcus occasionally produces a primary meningitis cannot be used as an argument, as is frequently done, in favor of the possibility that such an organism is capable of producing an epidemic of this disease, nor can the occurrence of several cases in one locality be so used. In view of the evidence advanced to show that the pneumococcus exhibits no special predilection for the meninges, it seems more rational to attribute the occurrence of several cases in one locality, thus simulating an epidemic, to chance.

In a series of eight cases of pneumococcic meningitis, we have never failed to find the existence of conditions which seemed competent to explain the localization of these organisms in the meninges. In six cases of meningitis complicating lobar pneumonia, the presence of a malignant endocarditis in three of them may be advanced to explain their localization in the meninges. In the three remaining cases, the existence of a chronic or an acute nephritis may have facilitated the lodgment of the organisms in these membranes. In two instances of pneumococcic meningitis, unassociated with lobar pneumonia, the existence of a well developed empyema of the accessory sinuses in one and of a thrombosis of the longitudinal sinus and chronic nephritis in the other, may serve to explain the meningeal localization of the pneumococcus. In enumerating the lesions associated with meningitis in these cases, we have selected only those which seemed most likely to act as determining factors. The frequent occurrence of chronic and acute kidney lesions is of interest. It raises the question whether alteration of the brain and meninges, brought about as a result of the kidney lesions, or induced by the same causative factors which produced the renal affection, may not be an important factor in determining in these structures a localization of an organism which otherwise shows no tendency to do so.

Netter[109] has shown that such a localization of the pneumococcus can be brought about in rabbits by previously cauterizing the leptomeninges. In the absence of such an initial injury, the organisms injected into the circulation fail to lodge in these membranes.

Councilman[108] reports a series of twenty-eight cases of pneumococcic meningitis, among which only three appeared to be instances of a primary involvement of the meninges. In one of these the existence of an acute nephritis may be advanced to account for the localization in the meninges.

Among one hundred cases of meningitis, due to a variety of causes, Libman[66] found only six which were due to the pneumococcus, and in these the involvement of the meninges was secondary.

If we base our opinion solely on the cases of pneumococcic meningitis, with which we are personally familiar, we find no good reasons for assuming a selective action for the meninges on the part of the pneumococcus.

We may add that the streptococcus, which has never been accused as a producer of epidemics of meningitis, occasionally gives rise to a primary meningitis. Councilman reports one such case and we are able to add another, although in our own case the existence of a streptococcic septicemia and an acute nephritis probably antedated the meningeal involvement. In view of the large number of cases of streptococcic septicemia without meningitis, we must assume the existence of very unusual conditions to account for the lodgment of the streptococcus in the meninges in this case.

If we turn to the literature on this topic we find no facts which seem competent in our opinion to disprove the assertions made above. In an excellent review of this subject, which we have verified in so far as the papers referred to were accessible, Schottmüller[68] arrives at the conclusion that only four epidemics have been recorded which were not due to the meningococcus. After covering practically the same ground we were led to similar conclusions. The epidemics referred to were reported by Bonomè[102] (sixteen cases), Panienski[104] (sixteen cases), Quadu[105] (eighty-six cases) and, finally, a small epidemic reported by Weichselbaum.[106] The report of Weichselbaum contains no data which would allow one to pass judgment on the nature of the organism concerned. According to this observer, the diplococcus pneumoniæ was the causal agent. When we consider that Weichselbaum allows for very considerable variations in its characteristics, it seems quite possible that organisms resembling the pneumococcus were accepted by him as such. As we have already stated, Weichselbaum accepts the organism described by Bonomè as a pneumococcus, an opinion which for reasons to be given presently we are not willing to endorse.

Finally, the question may be asked whether this observer was dealing with a genuine epidemic. The occurrence of several cases of a disease in one locality does not constitute an epidemic unless some relationship between the cases can be demonstrated or can be rationally assumed to exist. Whether such a connection was established, or could reasonably be assumed in this series, cannot be determined from Weichselbaum's report.

The epidemic reported by Quadu[105] was attributed by this observer to the diplococcus pneumoniæ. We were not able to obtain this paper in the original, and the data contained in the review were insufficient to determine the validity of this author's contention. We may, however, insert in this connection an argument advanced by Schottmüller against the pneumococcic nature of epidemics of meningitis. It is well known that a pneumococcic meningitis proves fatal in every instance, yet an epidemic of meningitis in which every victim of the disease died has not been recorded. If we return to the records of Quadu's epidemic, we find that out of eighty-six cases only sixty-seven proved fatal. Therefore, it seems unlikely that the pneumococcus was the causative factor in this epidemic.

In reviewing the descriptions given by Bonomè[102] and by Panienski[104] of the organisms isolated by them, the striking similarity to the streptococcus mucosus is at once apparent. Schottmüller[68] was the first to point out this similarity. For the following reasons we are unwilling to accept as proven his conclusions that these organisms are identical.

When we first isolated the bacterium which we employed for the animal experiments detailed above, we had little doubt that it was the streptococcus mucosus, and when we encountered Bonomè's report we were convinced of the identity of his organism with our own. Morphologically and culturally the bacteria isolated by Bonomè resembled our own very closely, and they also agree in so far as their pathogenic properties for rabbits are concerned. Bonomè's description of the clinical course of the disease induced in rabbits by the injection of his bacterium corresponds very closely to the observations made by us on rabbits similarly treated. The fact that this observer failed to establish the existence of a meningitis in the rabbits experimented upon may be attributed to his failure to examine these structures microscopically. No mention of such an examination is made in his report; and, as we have already stated, the macroscopic lesions in the brain of such animals are not sufficiently pronounced to lead one to suspect the existence of a meningitis. We believe that the clinical manifestations of meningeal involvement in his animals warrants us in assuming the existence of such lesions.

The organism isolated by Panienski also appears to be identical with that isolated by Bonomè.

As we have already indicated, the encapsulated streptococcus isolated by us did not survive long enough to enable us to determine its relationship to the streptococcus mucosus. We are therefore not in a position to claim or disclaim the identity of these organisms.

In our efforts to recover a similar one for further experiments, we isolated three streptococcus mucosus strains, and obtained a fourth from Drs. Norris and Flournoy, which was recovered from the blood in a case of meningitis. Numerous experiments on rabbits were undertaken, and these strains all failed to reproduce either the clinical or anatomical picture of meningitis. While many of the animals succumbed to the injections, the autopsy revealed the lesions of a septicemia and frequently a purulent peritonitis; but in not a single instance was an involvement of the meninges demonstrable. These observations prompt us to withhold an opinion concerning the relationship of these organisms until further evidence is available.

If we apply the original propositions which formed the basis for these arguments to the organism isolated by Bonomè, we may say that it possesses the peculiarities which enable it to produce, unaided by other factors aside from a predisposition on the part of the infected animal, a primary and predominant involvement of the meninges, and may therefore produce epidemics of meningitis. It exhibits a special predilection for the meninges when introduced into rabbits as shown by the fact that it lodges by preference in these membranes when injected into parts removed from the central nervous system. The fact that the disease thus produced in rabbits closely resembles that noted by Bonomè in spontaneous infections in man suggests that here also the organism does not gain access to the brain by direct channels. The disease in man, according to this observer, usually begins very suddenly, which in our opinion is incompatible with a direct mode of infection. It follows, therefore, that this organism also displays a selective action for the meninges of man when it gains access to the body.

In conclusion we may say that thus far only two organisms have been encountered which exhibit a special affinity for the leptomeninges of man, thus distinguishing them from the other known pathogenic bacteria. These are the meningococcus of Weichselbaum and an organism closely resembling the streptococcus mucosus. A review of the literature also leads us to conclude that these are the only organisms whose causal connection with epidemics of meningitis has been established. The causal relationship of the diplococcus pneumoniæ to epidemics of meningitis has not been established, moreover such a relationship seems unlikely in view of the fact that this organism ordinarily shows no disposition to involve the leptomeninges.

General summary and conclusions concerning the mode of dissemination and the mode of origin of epidemic cerebrospinal meningitis:

1. The meningococcus is transmitted directly through the medium of the air from individual to individual. An authentic instance of indirect infection has not been recorded. Moreover, the hypersensitiveness of the meningococcus to drying renders such a mode of infection improbable.

2. The most important part in the dissemination of this disease is played by so-called meningococcus carriers. During an epidemic their number greatly exceeds the number of those who actually acquire the disease.

3. The occurrence of adult germ carriers, and the fact that the majority of individuals are naturally immune to infections with this organism, explains many of the peculiarities of epidemics of this disease.

4. The almost constant presence and the early appearance of the meningococcus in the respiratory passages of individuals suffering from this disease indicate that they gain access to the body by these channels. The fact that the organism is demonstrable in the throats of individuals who come in contact with meningitis patients, combined with the fact that the meningococcus is not a normal inhabitant of the human throat and is only rarely so encountered during non-epidemic periods, may be advanced in support of this view. The probability that it is not sufficiently resistant to pass through the stomach without being destroyed may be mentioned in opposition to the contention that it may gain access to the body by way of the intestinal tract.

5. The fact that repeated examination of the nasopharynges of individuals occupying wards harboring cases of meningitis (children) failed to reveal a single germ carrier may be mentioned in support of the relative innocuousness of children actually suffering from the disease.

6. The experiments designed to determine whether the organisms were excreted by way of the nasopharynx yielded negative results.

It must be admitted, however, that our experiments are not wholly competent to establish this point.

7. The question concerning the paths selected by the meningococcus in reaching the brain cannot be definitely answered with the data in hand, although all the evidence obtainable is in favor of a hematogenous origin of the disease. In support of this view we may mention the early generalization of the bacteria as shown by the anatomical findings in individuals who succumbed within twenty-four hours, the early appearance of the meningococcus in the blood in some instances, and the simultaneous occurrences of symptoms referable to the brain and to structures more or less removed from the central nervous system. In further support of this view we have attempted to show that the mode of onset of this disease is incompatible with the assumption that it gains access to the brain by direct channels.

8. Finally, we have advanced experimental evidence in favor of a hematogenous origin of meningitis.

While these experiments were undertaken with organisms not related to the meningococcus, the fact that the disease induced in rabbits by intravenous injections of these organisms resembled that occurring spontaneously in man in all essential particulars, suggests that the mode of origin in both diseases is identical. The further conclusions which we have drawn from these experiments are:

First, That the organism in question exhibits a special predilection for the meninges. The point of entry, therefore, is immaterial. The organism once gaining access to the circulation tends to lodge in the meninges.

Second, That the predominant involvement of the meninges covering the base of the brain is not determined by the point of entry. We are therefore inclined to question the validity of the contention that the chief lesions are found at the base of the brain in epidemic meningitis in man, because the organisms gain access to the cranial cavity through the base of the skull and first involve this portion of the meninges.

9. Concerning the relationship of other bacteria to epidemics of meningitis, we have arrived at the conclusion that only one other organism is capable of producing epidemics of this disease, namely, the encapsulated streptococcus of Bonomè. This conclusion is based on a review of the literature and on an analysis of the pathogenic properties of the organisms which have been known to cause epidemics of this disease. Both the meningococcus and the encapsulated streptococcus of Bonomè exhibit a special predilection for the meninges. The pneumococcus, which it is claimed not infrequently produces epidemics of meningitis, bears no such relationship to the meninges. We believe, therefore, that this organism is not concerned in the production of epidemics of meningitis. We are unable, with the data in hand, to answer the question concerning the relationship of Bonomè's coccus to the streptococcus mucosus.

[In conclusion we wish to thank Dr. Charles Norris, Dr. A. M. Pappenheimer, Dr. Thomas Flournoy, Dr. Frederick Humphries, and Dr. I. Strauss for cultures of the meningococcus and gonococcus furnished by them.]

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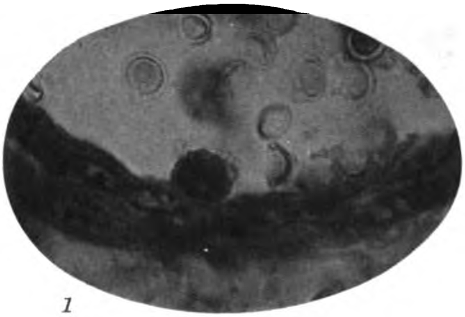
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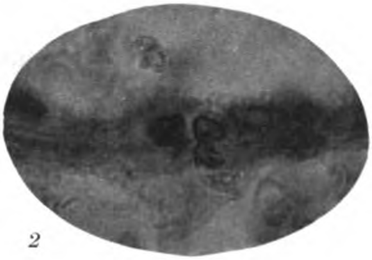
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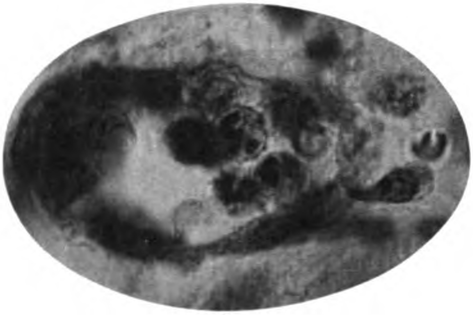
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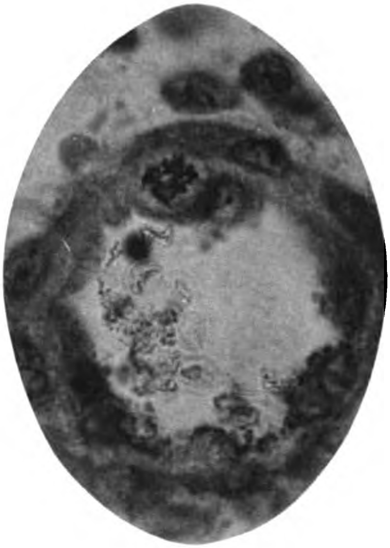
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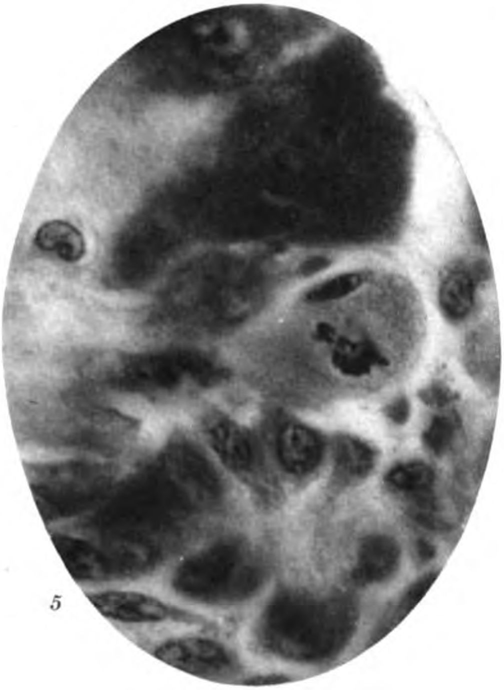
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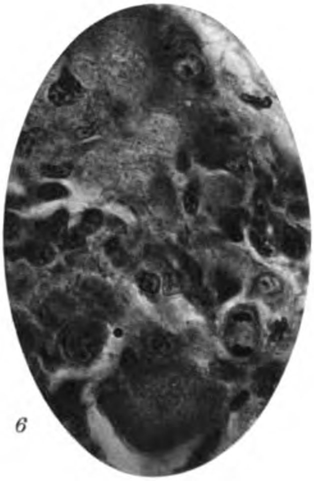
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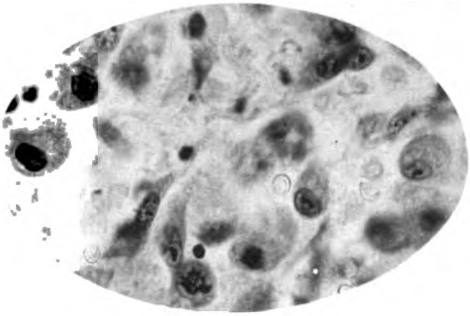
Forbes.

Giant-cells.



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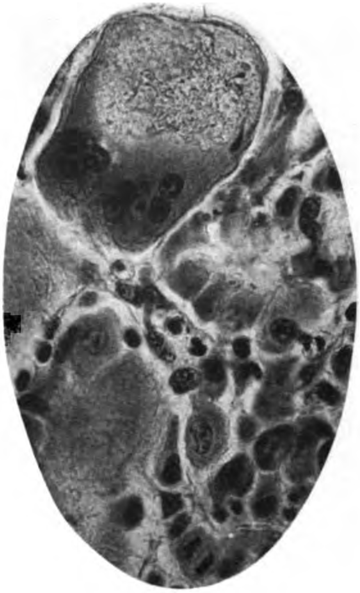

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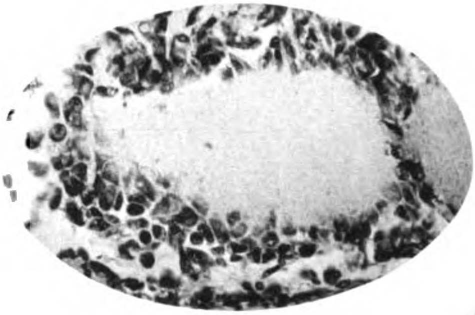

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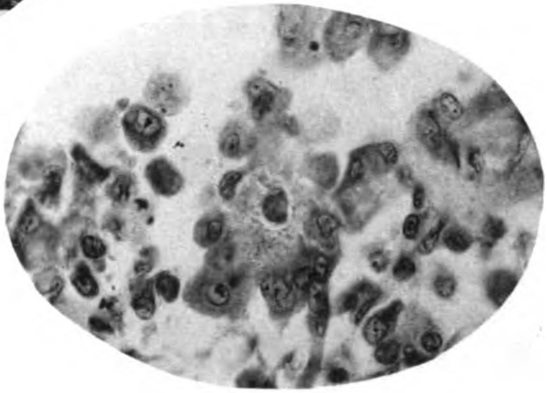

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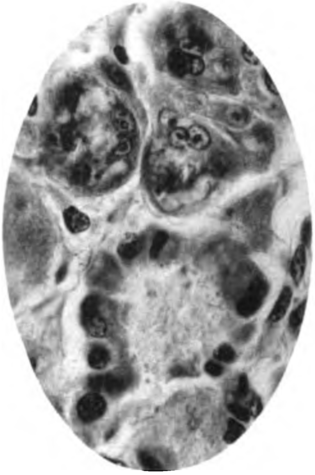

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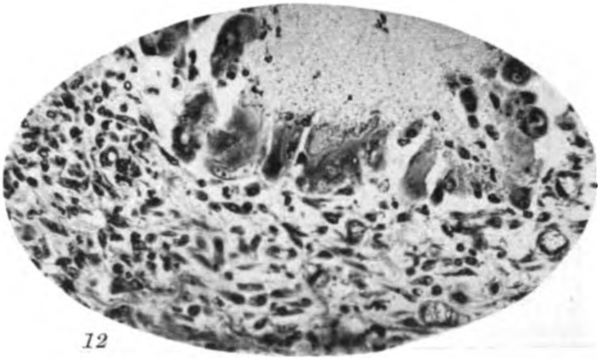

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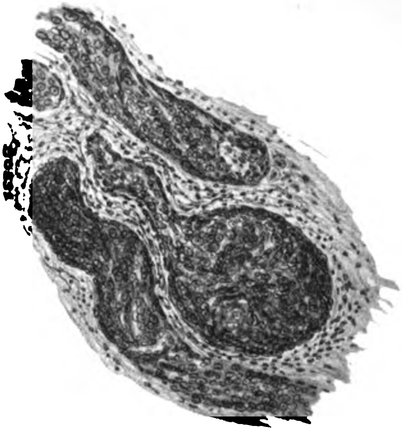
Forbes. Giant-cells.



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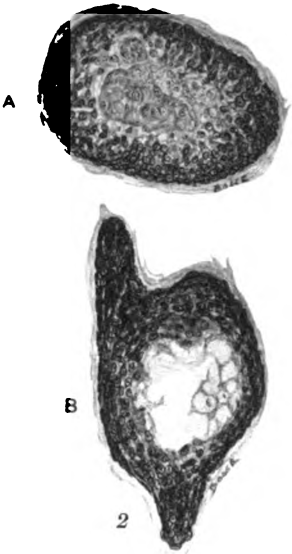

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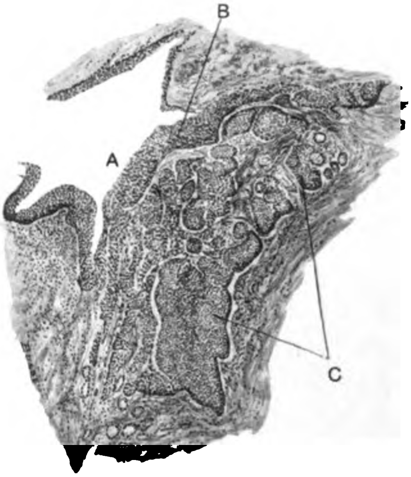

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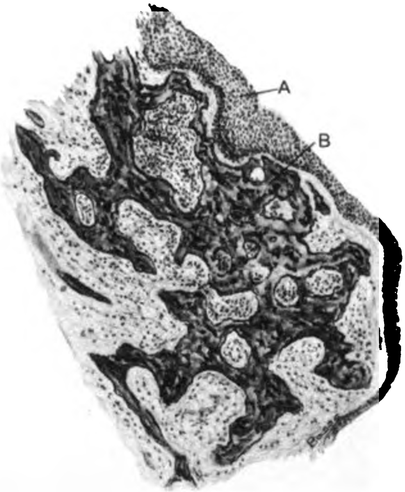

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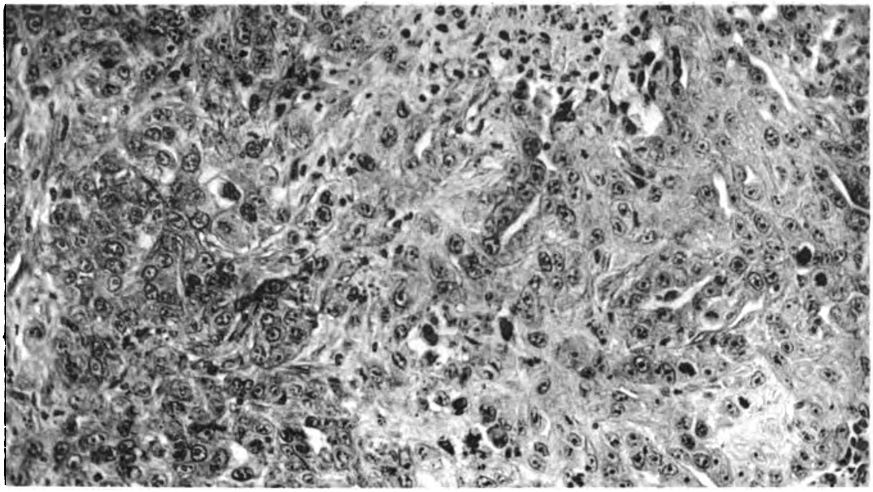

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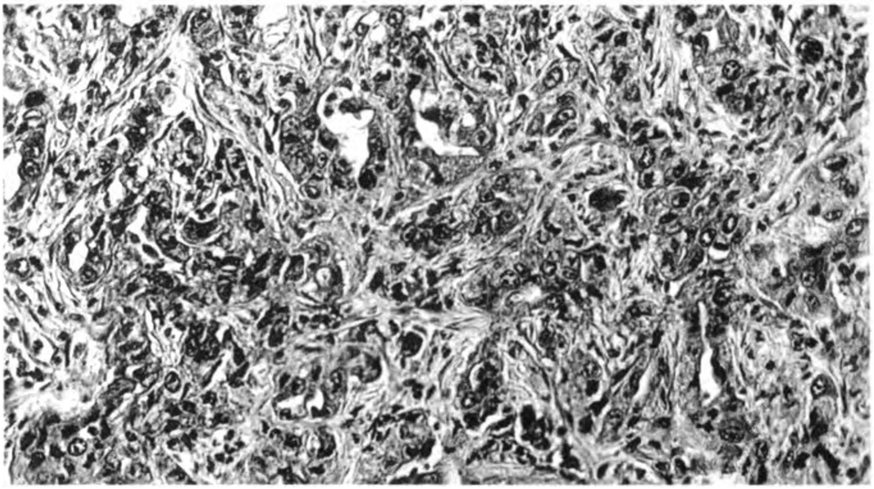

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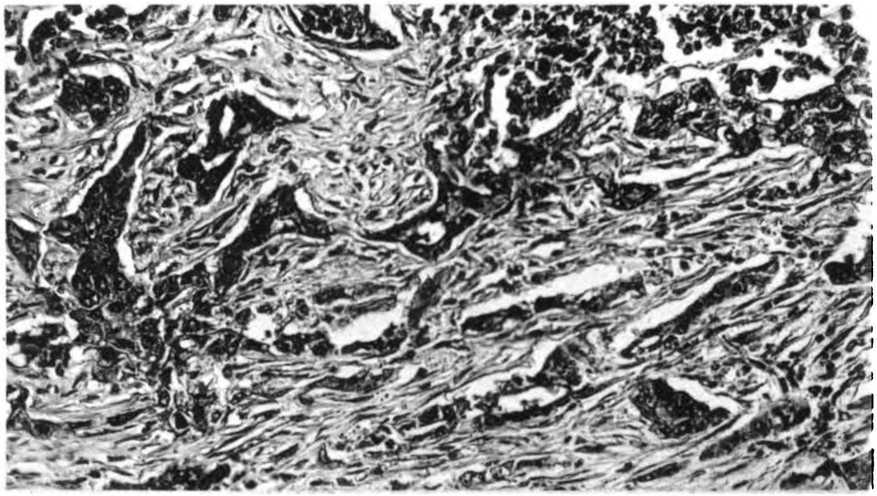
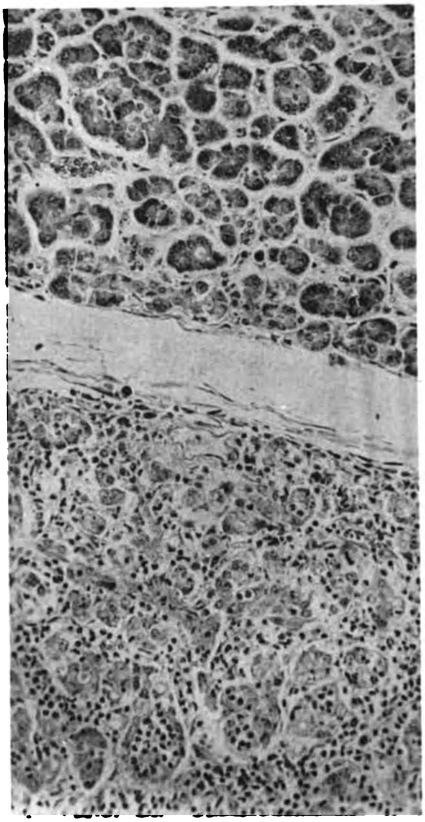


3

Gay.

Cancer of rat.



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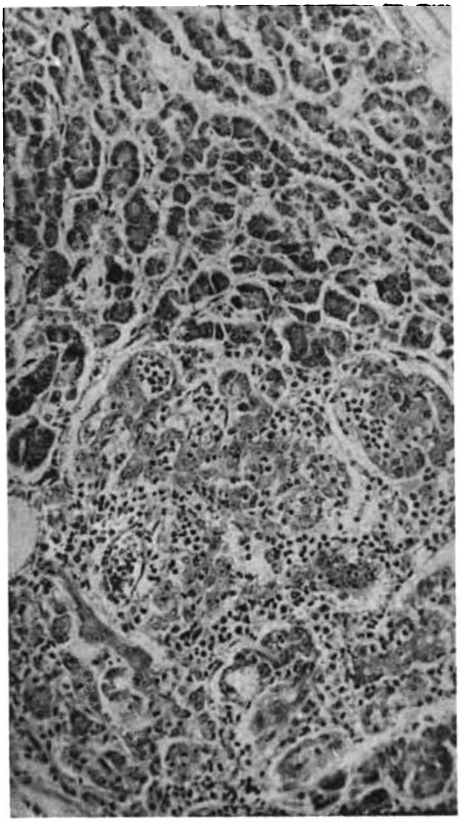

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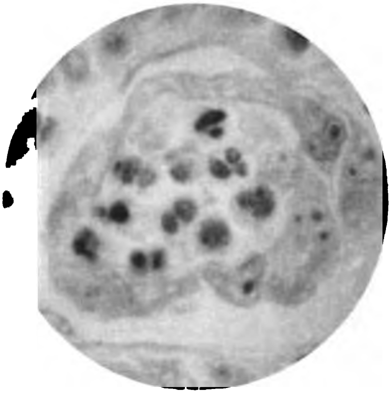

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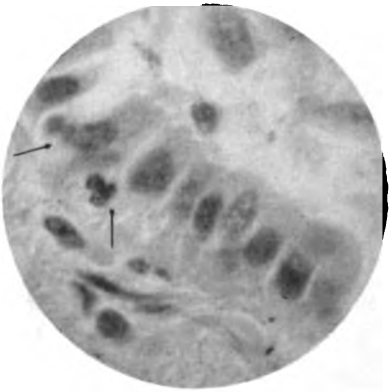
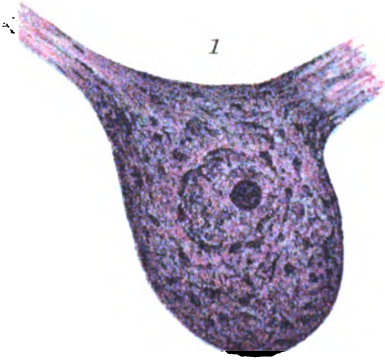


4

C. Frothingham, Jr.

Acute Pancreatitis.

1



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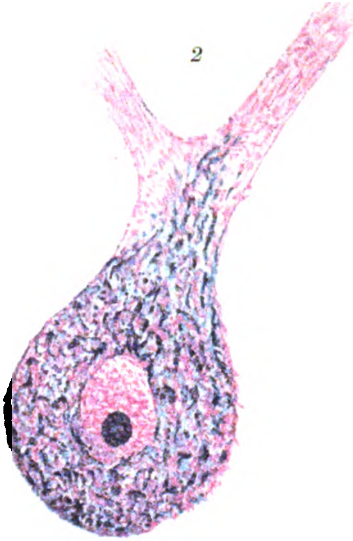

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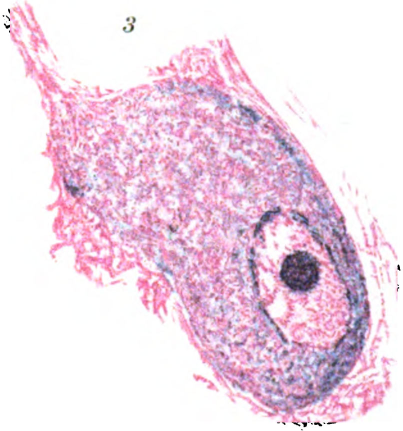

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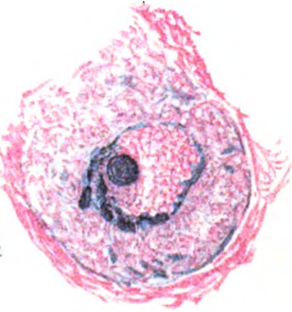

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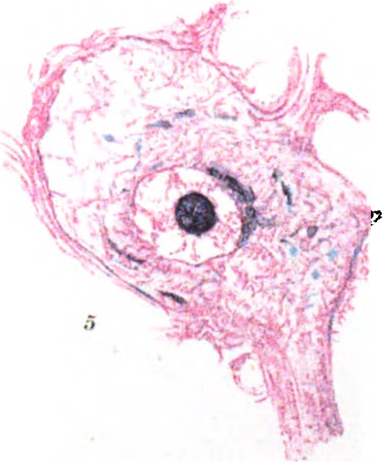
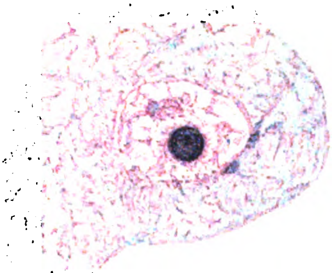


Dolley and Crile. Surgical shock.



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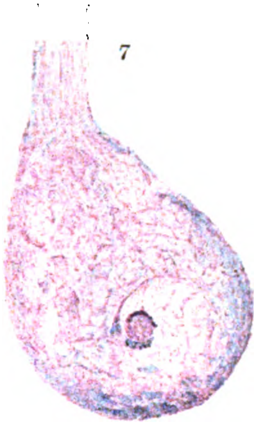

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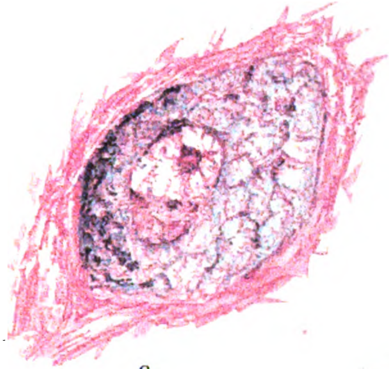

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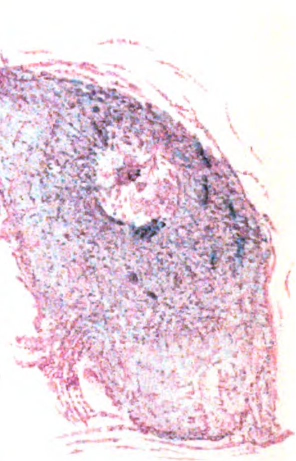

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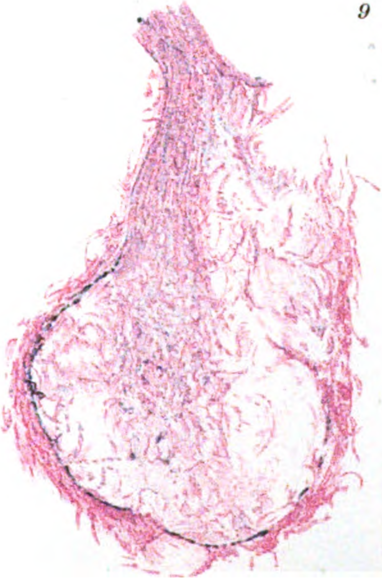

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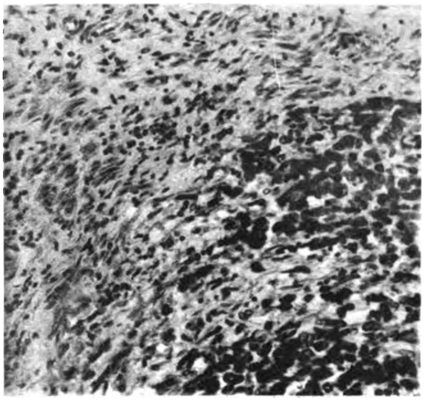
Dolley and Crile.

Surgical shock.



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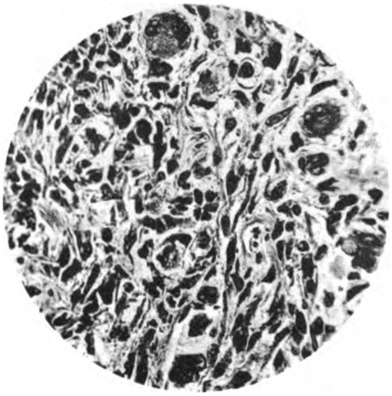

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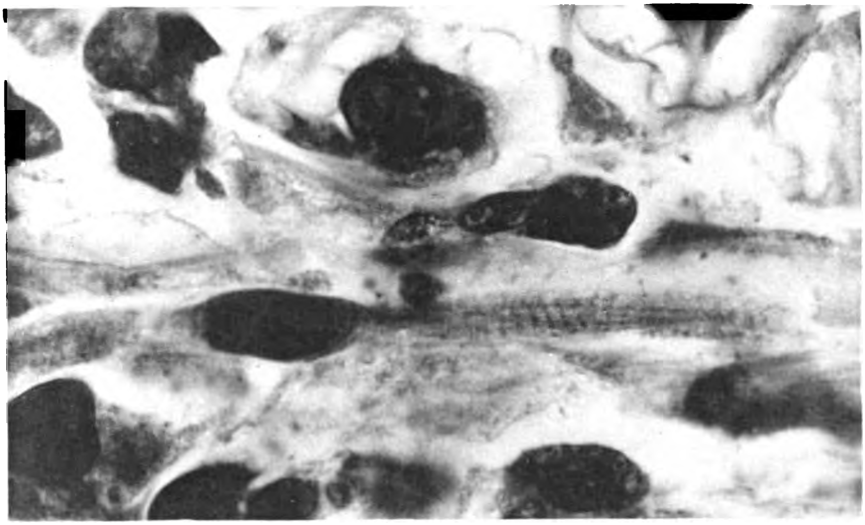

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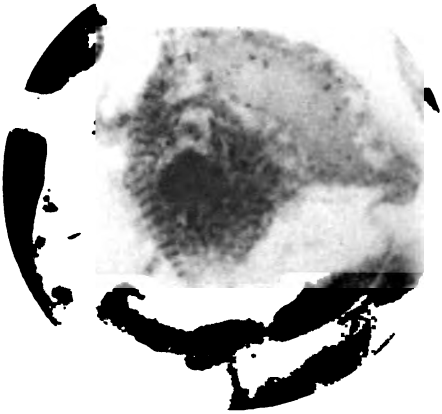

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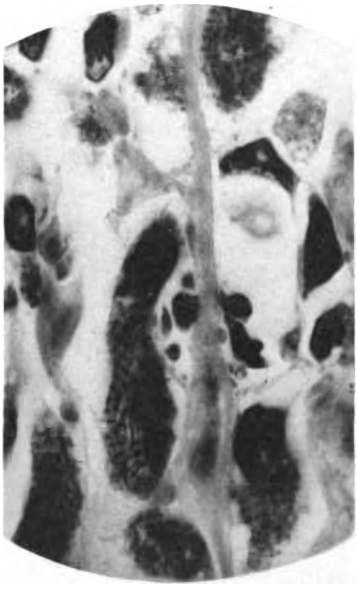

5

Robertson.

Rhabdomyosarcoma.